THE EFFECT OF REDUCTION, TRYPSIN DIGESTION AND DE-GLYCOSYLATION OF SALIVARY MUCINS IN THE INHIBITION OF HUMAN IMMUNODEFICIENCY VIRUS TYPE 1

MISS ELLIS C.T TSETSE

PRESENTED FOR THE DEGREE OF

MASTER OF SCIENCE IN MEDICINE (SURGERY)

DEPARTMENT OF SURGERY

FACULTY OF HEALTH SCIENCES

UNIVERSITY OF CAPE TOWN

FEBRUARY 2019

Supervisor: Professor Anwar Suleman Mall

DECLARATION

This work was completed under the supervision of Professor A. S. Mall, from 2017 to 2018 in the Division of Surgical Research, University of Cape Town.

I hereby certify that the following study is my own work. I have used the Virology Journal output style for citation and referencing (RefWorks Web Based Bibliographic Management Software employed by the University of Cape Town). Each contribution to, and quotation in this thesis from the work(s) of other people has been attributed and has been cited and referenced.

Signature: Ellis C.T Tsetse Student No: TSTELL001

Ellis Tsetse

March 2019

Signature: Signed by candidate

Professor A. S. Mall

March 2019

ACKNOWLEDGEMENTS

I would like to thank my supervisor Professor A. Mall for his dedication to the project, for providing enthusiasm and motivation and believing in my work.

I would like to extend my thanks and appreciation to the following people, without whom this project would not have been possible or such a pleasure.

Dr J. Dorfman and the ICGEB, for his help and willingness in supervising the Neutralisation assay and for our discussions about great ideas, which gave me much motivation. PhD student, Samuel Kariuki for constant guidance and advice. Your kindness was much appreciated.

Marilyn Tyler and Lizeette Fick for their help and direction not only in the laboratory but also with life lessons! For their kind and endearing nature and the time and effort they gave to me to make my time in the department so valuable and enjoyable. I would like to thank them very much for the proof reading of my thesis and for their encouragement with presentations for the project. Thank you for everything!

Vuyowethu Siyo and Baxolele Mhklekude, my colleagues in the lab, for being such wonderful friends to me, and for all the advice they gave me for this project.

I would like to thank the MasterCard Foundation and the PRF at UCT for kindly aiding with funding for my studies. I am so deeply grateful for this support.

My mother (Patricia Tsetse) and my sister(Jane Kundishora) who have believed in me throughout this project. Thank you for being a force in my life and for fuelling me up every time I could not go on . l am because of your prayers. Thank you for everything!

I would like to thank my partner (Tafadzwa Makwangudze) and my friends (Vimbainashe and Dave Adekoya, Josiah Makuni, Ruvimbo Nyaruviro, Grace and Lesogo khoza, Rumbidzai and Nevanji Mutumbwa, Petros and Daniela Dube, Victoria Basopo and Khanyisile Chiganze) who have helped me to pursue my degree, and my dreams, and for the endless good times we have shared. Thank you for your thoughts.

God, without whom, none of this would be possible. Thank you for being such a great force in my life, and I pray for your will to be done in my future.

ABSTRACT

In 2017, 36.7 million people worldwide were living with Human Immunodeficiency Virus (HIV) and of that total, 1.8 million people were new infections. Sub-Saharan Africa was recognized as the most afflicted regions worldwide accounting for 26 million people, 68%, living with HIV. The difficulty in fighting this epidemic has raised the urgent need for research exploring ways in which HIV transmission can be curbed worldwide.

Our laboratory previously showed that crude saliva and purified salivary mucins (MUC5B and MUC7) inhibit HIV-1 infection *in vitro*. However, it is not known whether the specific arrangement of mucin carbohydrate residues is important for mucin interactions with HIV-1, or if the negative charge afforded by sialic acid and sulfated sugars allows binding to viral receptors. While giving some important insight into the mechanism of HIV inhibition, we hope that this study will determine the minimum peptide chain length and structure of a gel forming mucin that retains the anti-HIV activity. In addition, we aim to determine if the reduced salivary subunits and trypsin digested fragments retained this inhibitory activity against HIV-1.

Saliva was collected and stirred overnight in 6M guanidine hydrochloride with 10mM Na2HPO4, 10mM EDTA, 1mM PMSF and 5mM NEM. Salivary mucins (MUC5B and MUC7) were purified using caesium chloride ultracentrifugation and separated on a Sepharose CL-4B column. Thereafter, mucin rich fractions were either reduced with 10mM dithiothreitol (DTT) or proteolytically digested with 0.25% trypsin. The resultant fractions were dialysed and freeze dried. Slot blots were used to determine the identity of the void volume (V_o) fractions and the included volume (V_i) fractions which were identified as MUC5B and MUC7 respectively. The V_o and V_i fractions were subjected to 4-20% sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) to determine the size and mucin concentration. In addition, mucin oligosaccharides were enzymatically removed using the de-glycosylation kit (EDEGLY) purchased from Sigma Aldrich (UK). Following this, all mucin lyophilized aliquots were tested for cell cytotoxicity using an MTT assay. This was then followed by a neutralisation assay which uses HIV-1 env pseudo virus

(DU422.1 and YU2 subtype C and subtype B respectively) and a luciferase reporter gene involving modified TZM-bl/JC cells was used to test the inhibitory activity of the test samples.

Comparison of the anti-HIV activity of crude saliva, MUC5B and MUC7 against the DU422 virus showed that both crude and purified saliva indeed inhibits the infection of the DU422.1 pseudo-virus strain to TZM-bl/JC cells (Kruskal-Wallis, $p=0.00025$). MUC5B was more potent in inhibiting the DU422 virus as compared to crude saliva and MUC7 (Mann-Whitney U, $p=0.0227$ and $p=0.0195$ respectively). Furthermore, no difference was observed in inhibiting the DU422 virus by MUC7 and crude saliva (Mann-Whitney U, $p=0.128$). While the three cohort of samples did inhibit the YU2 pseudo virus (Kruskal-Wallis, $p=0.0078$), MUC7 showed a higher inhibition compared to MUC5B and crude saliva (Mann-Whitney U, $p=0.0341$ and $p=0.176$ respectively). A significant difference in the inhibition of the YU2 virus was detected between MUC7 and crude saliva (Mann-Whitney U, $p=0.0031$). In addition, reduced and digested salivary fragments inhibited both viruses suggesting the possibility that even when the gel forming properties of mucins are compromised, mucins still retain their inhibitory activity.

Interestingly, the removal of oligosaccharides showed MUC5B as the most potent mucin in the inhibition of both DU422 and the YU2 pseudo virus (Kruskal-Wallis, $p=0.0312$). De-glycosylated MUC7 displayed minimal inhibition against the YU2 and DU422 virus suggesting that oligosaccharides are important for maximal inhibition. Furthermore, this highlights that the mechanism through which mucins inhibit viruses involve glycans.

In conclusion, the results of this study suggest that MUC5B can be harnessed and used as a core component of a microbicide which can be used to prevent HIV transmission. Its extensive glycosylation compared to MUC7 makes it a better candidate for this anti-HIV-1 inhibitory activity.

ABBREVIATIONS

AIDS	Acquired immune deficiency syndrome
AMPS	Ammonium persulphate
ARV	Antiretroviral
CsCl	Caesium chloride
CCR5	C-C-Chemokine receptor type 5
CD4	Cluster of differentiation 4
CXCR4	CXC Chemokine receptor type 4
DMEM	Dulbecco's Modified Eagle's Medium
DC-SIGN	Dendritic cell-specific intercellular adhesion molecule 3 grabbing non intergrin
dH2O	distilled water
DTT	DL-dithiothreitol
EDEGLY	Enzymatic Protein de-glycosylation kit
EDTA	Ethylenediaminetetraacetic acid
Env	Envelop
FACTS 001	Follow on African Consortium for tenofovir studies 001
FBS	Fetal bovine serum
FUC	Fucose
GalNAc	N-acetylgalactosamine
GlcNac	N-acetylglucosamine
Gp	Glycoprotein
GuHCL	Guanidinium chloride

HAART	Highly Antiretroviral Therapy
HCL	Hydrogen Chloride
HIV	Human Immunodeficiency Virus
HRPO	Horse radish peroxidase
HSV	Herps simplex virus
IAA	Iodoacetamide
IC50	Half maximal inhibitory concentration
ICAM-1	Intercellular Adhesion Molecule 1
ICGEB	International Centre for Genetic Engineering and Biotechnology
Ig	Immunoglobulin
IN	Integrase
kDa	Kilodalton
KZN	Kwazulu Natal
L	Litre
LC50	50% lethal concentration
PBMC	Peripheral blood Mononuclear cells
ER	Endoplasmic Reticulum
LC ESI-MS	Liquid chromatography electrospray ionisation mass spectrometry
VOICE	Vaginal and oral interventions to control the epidemic
M	Molar
Mbar	Millibars
Mg	Milligram
Mins	minutes
ml	Millilitre
mM	Millimolar

MTT	Thiazolyl blue trazolium bromide
MUC	Mucin
NEAA	Non-Essential Amino ACID
NEM	N-Ethylmaleimide
NeuAc	N-acetylneuraminic acid
NH_4HCO_3	Ammonium bicarbonate
NaCl	Sodium chloride
NHLS	National Health Laboratory Services
Nm	Nanometre
NTRI	Nucleotide reverse transcriptase inhibitor
PAGE	Polyacrylamide gel electrophoresis
PAS	Periodic acid Schiff
PBS	Phosphate-buffered saline
PBST	PBS tween-20
PMSF	Phenylmenthanesulfonylfluoride
PR	Protease
PRI	Protease Inhibitor
PRP	Proline rich protein
Rpm	Revolutions per minute
RT	Reverse Transcriptase
SAG	Salivary Agglutinin
SDS	Sodium dodecyl sulphate
sIgA	Secretory immunoglobulin
STI	Sexual Transmitted infection
TAE	Tris-acetate-EDTA
TBST	Tris buffered saline-tween

TEMED	N, N, N, N'-tetramethylenediamine
TFV	Tenofovir
Tris	Hydroxymethyl aminomethane
Tris-HCl	Tris-Hydrochloric acid
DEAE	Dextran
RLU	Relative light unit
UCT	University of Cape Town
UNAIDS	United Nations Programme on HIV/AIDS
V_o	Void Volume
V_i	Included Volume
w/v	Weight/volume

Table of Contents

List of Figures

List of Tables

CHAPTER ONE: GENERAL LITERATURE

1.1 The Human Immunodeficiency Virus Epidemic

In 2017, the United Nations Joint Programme on Human Immunodeficiency Virus /Acquired Immunodeficiency Syndrome (UNAIDS) reported that approximately 36.7 million people worldwide were living with Human Immunodeficiency Virus (HIV) and of that total, 1.8 million people were new infections (UNAIDS, 2017). About 21,7 million people worldwide were accessing antiretroviral treatment in 2017, and despite the availability of treatment, 1.2 million people died globally of Acquired Immunodeficiency Syndrome (AIDS) related illness (UNAIDS, 2017).

Sub-Saharan Africa was recognised as the most afflicted regions worldwide accounting for 26 million people ≅ 68%, living with HIV. More than half of the affected individuals were women. By the end of 2017, 7.1 million people in South Africa were living with HIV with around 270 000 new infections. Of these newly infected cases, only 54% had access to antiretroviral therapy drugs (UNAIDS 2017). By year-end of 2016, the South African National Aids Council released the HIV provincial data which highlighted Kwazulu Natal (KZN), Gauteng and Eastern Cape as the provinces with the highest burden of HIV (National Department of Health 2015). Furthermore, when the statistics were broken down according to age and gender, the burden of HIV was high amongst woman between the age of 20-34 (National Department of Health 2015).

Regardless of the steady increase in the number of people living with HIV in South Africa from 2016 to 2017, the number of infected individuals still remains high as compared to other countries in the world. This increase in infection may be due to an increase in unsafe sexual practices such as multiple partners, no condom use or no condom adherence in infected individuals (UNAIDS 2013). South Africa currently struggles with this epidemic and effective treatment and preventative strategies are required to reduce

the prevalence and incidence of HIV. With that being said, this indicates a necessity for research exploring ways in which HIV transmission can be curbed in the region.

1.2 HIV Taxonomy and Characteristics

HIV is part of the Retroviridae family. This class of envelope viruses infects the cells by using an enzyme called reverse transcriptase, which transcribes viral RNA into DNA. This viral DNA is then integrated into the human genome for viral replication to occur. Furthermore, HIV is classified into the lentiviruses genus which is characterized by long periods of incubation which usually results in chronic illness (Narayan and Clements 1989).

Based on the genetic characteristics and difference in viral antigens, HIV is divided into two main groups mainly HIV type 1 and HIV type 2 usually written as HIV-1 and HIV-2 respectively (Sharp.et al. 2005). The evolution of these viruses differ. HIV-1 is thought to have evolved from a chimpanzee in Central Africa while HIV-2 is thought to have evolved from the West African Mangabeys (Hahn et al. 2000; Sharp.et al. 2005; Lemey et al. 2003). HIV-1 is more prevalent and progresses faster to AIDS whereas HIV-2 is less virulent and has a slower progression (Nyamweya et.al 2013). Phylogenetic analysis has shown that HIV-1 is further divided into the three main subtypes based on their transmission mainly Major (M), Outlier (O), and non-Outlier (N) (Cohen et al. 2008). The most common HIV-1 group is the M group which comprises of 13 subtypes namely A1, A2, A3, A4, B, C, D, F1, F2, G, H, J and K (Cohen et al. 2008). These subtypes contain distinct different DNA sequences which differ by 15-20 % (McChutan 2000). Most individuals in Australia , Asia and Europe are usually infected with the subtype B of the M group HIV-1 virus while the subtype A, C and D are concentrated in Africa (Teixeira et al. 2011). HIV-2 consist of different subtypes from A-E with subtype A and B being the most prevalent (Chen et al. 1997).

Different HIV-1 M strains have been spreading leading to global epidemic. Subtype C accounts for 48% of the total HIV-1 infections globally and it has the highest prevalence rate in South Africa. In 2003, Kwazulu Natal was one of the most affected provinces which had the highest infection rate of the subtype C virus (Gordon et al. 2003). It is still one of the most affected provinces in South Africa.

1.3 The Structure, Genome and Morphology of HIV-1

HIV is an enveloped virus and during its replication process, it buds off with some of the hosts cell membrane which allows the virus to replicate without eliciting an immune response (Engelman and Cherepanov 2012). The genetic material of this virus consists of two identical single stranded RNA molecules which contain nine viral genes namely gag, pol, env, tat, rev, nef, vif, vrf and vpu (Rajarapu 2014). Gag, pol and the env viral proteins are the structural proteins while the rest of the viral proteins are involved in regulating viral replication and infection. The genomic RNA together with the viral proteins are enclosed by the viral nucleocapsid protein (Rajarapu 2014).

Reverse Transcriptase (RT), Integrase (IN) and Protease (PR) are the three major enzymes that are involved in the replication cycle. These enzymes are encoded by the pol gene and are cleaved products of the Gag-Pol precursor polyprotein (Rajarapu 2014). The enclosing capsid called the p24 is also a product of the gag precursor together with the matrix often called the p17 protein. All these proteins are enclosed within a lipoprotein rich viral envelope which consists of host membranes that are incorporated during the budding off process to infect new cells (Sandquist and Krausslich 2012; Engelman and Cherepanov 2012). This viral envelope consists of a trimer which is composed of an external glycoprotein called gp120 and a transmembrane protein gp41 which anchors the heterodimer complex (Engelman and Cherepanov 2012; Sandquist and Krausslich 2012; Rajarapu 2014). These two proteins are encoded by the env viral protein and are cleaved products of gp160.

The HIV-1 virion is circular in shape, approximately 80nm-100nm in diameter and consists of a lipid bilayer that comes from the host cell membrane. The lipid bilayer consists of cellular membrane proteins which are derived from the cell membrane of the hostand these include Major histocompatibility complex 1 and 2 (MHC1 and MHC 11) proteins and the intercellular adhesion molecule (ICAM-1) (Briggs et al. 2003; Rubbert et al. 2007; Turner and Summers et al. 1999).

1.4 Sexual Transmission of HIV In Host Cells

Dendritic cells, CD4 T cells and macrophages are susceptible cells which are usually found in the genital and corelectal mucosae where exchange of bodily fluids usually occurs (Haase 2011). HIV-1 targets cells that display Cluster of differentiation (CD4) receptors on their surfaces in particular CD4 T lymphocytes (Maddon et al.1986). These CD4 cells have co-receptors that are either CCR5 or CXCR4. The CCR5 co-receptor is commonly utilized during HIV transmission and viruses that are macrophage-trophic and use this receptor are known as R5 viruses (Levy 1996; Bergery 1999). X4 viruses use the CXCR4 coreceptors to infect CD4 T cells (Berger et al. 1999; Tersmette et al. 1998).

The first phase of the viral replication begins with the binding of the virus to the host cells. This interaction occurs between the gp120 extracellular domain of the virus and the CD4 host cell receptor found on the surface of the target cells which also includes macrophages, dendritic cells (Maddon et al. 1986). The binding of the virus to the CD4 receptor results in a structural change which exposes the CCR5 and CXCR4 coreceptors which results in a high affinity bond being created between either the co-receptors or the gp120 extracellular domain (Pancera et al. 2010; Haase 2011; Klasse 2012). In addition, these receptor binding changes induces some conformational changes in the gp41 protein resulting in the formation of the viral/target cell membrane complex (Pancera et al. 2010). The virus particles enter the host cell cytoplasm and it releases its viral RNA which is converted into double stranded viral DNA by reverse transcriptase enzyme. This process tends to be highly error prone which gives rise to many mutants that are drug resistant (Yu et al. 2003). The viral DNA then become integrated in the nucleus of the host

cell genome by the enzyme integrase resulting in a nucleoprotein complex that consists of viral DNA and Integrase (Klasse 2012, Chen et al. 2012). During the latent stage of HIV transmission, the proviral DNA usually remains dormant and any efforts to eliminate the plasma viremia by highly active antiretroviral drugs (HAART) may still allow the virus to persist creating a major barrier on how to eradicate this virus (Finzi et al. 1999).

1.5 Transmission Of HIV-1

Sexual intercourse is one of the most prevalent route for HIV-1 transmission. Interestingly, other modes of transmission such as mother to child transmission have become common routes for HIV acquisition (Royce et al. 1997). The rate at which transmission occurs depends on a number of factors such as the type of sexual activity, presence of ulceration or inflammation in the genitalia or even circumcision status (Powers et al. 2008). Moreover, the concentration of HIV and the number of HIV infected cells in the relevant bodily fluid has to be high for transmission to occur (Cohen et al. 2008). Viral shedding of HIV has been found in blood, semen, breast milk, saliva, seminal fluid, rectal fluid, and cervicovaginal fluids all of which form a media through which the virus can be transmitted (Pudney et al.1992; Zuckerman et al. 2004; Pilcher et al. 2009).

The skin provides an excellent barrier against viral infection as it does not contain any cells susceptible to HIV. However, compromising the architecture of the skin via physical impairment or wounds creates entry points for the virus (Luizz et al. 1996). Due to the target cells present in the bloodstream, blood to blood contact is clearly a highly efficient route through which HIV transmission occurs (Ho, Moudgil and Alam 1989; Liuzz et al. 1996). Blood to blood contact via sharing of needles is another mode through which HIV is transmitted. Abdala et al. (2000) demonstrated that HIV could remain viable in a needle for up till 42 days depending on the environmental conditions providing another route for viral infection.

The primary route through which HIV is transmitted is via the mucous membranes. These membranes line the epithelium of the internal tracts of the body thereby creating a

barrier against pathogens and bacteria. The delicate nature of these membranes makes them more susceptible to damage and consequently lead to HIV-infection. Since these mucous membranes are more delicate than the skin and also contain a significant number of dendritic cells which are target cells for HIV, no damage to the membrane is required for HIV transmission to occur. For example, Wu and KewalRamani (2006) demonstrated that dendritic cells in the vaginal epithelium and the rectal mucosa can bind HIV particles from the lumen and transport it to CD4 + cells providing a route for HIV to disseminate throughout the immune system (Wu and KewalRamani 2006).

Globally, HIV transmission is mainly via heterosexual intercourse . The chances of contracting HIV are greater when the transmission is passed from a man to a woman rather than from the woman to a man (Durieux-Smith and Goodman 1992; Pettifor et al. 2005). The increase in susceptibility is because of the larger surface area of the vagina that consists of HIV targeted cells and a higher viral concentration in semen as compared to vaginal secretions. Young girls are also at a higher risk of getting infected because the genital mucosa has not yet fully developed (Muula 2008; Ramjee and Daniels 2013). Anal intercourse presents a higher risk of transmission as compared to vaginal intercourse because of the high viral loads that are present in the rectal fluids. In addition, the epithelium of the anus is not as elastic as the vaginal mucosa which makes it very susceptible to tears and abrasions which creates entry points for the virus (Zuckerman et al. 2004).

The transmission of HIV from mother to child accounts for approximately 30% to 40% of the majority of infant infections and transmission which occurs during birth, via breastfeeding or when the child is *in utero* (Dickover et al. 1996; Lehman et al. 2013; Mofenson et al. 1999). The risk of transmission depends on a number of factors, but a high maternal virus load has been demonstrated to cause a major risk (Lehman et al. 2013, Mofenson et al. 1999, Dickover et al. 1996; John et al. 1996). In the absence of treatment, the risk of mother to infant transmission via breastfeeding accounts for approximately 16% (John-Stewart 2008). The provision of a single dose of nevirapine during labour to the mother can significantly decreases the risk of transmission rate by

50% while the taking of antiretroviral drugs during pregnancy and breastfeeding also reduces the risk by 5% (Shapiro et al. 2010; Lancet Infect Dis 2010). Ziegler et al. (1985) recorded the first ever case of mother to child transmission which was observed in an HIV-negative woman who had given birth via caesarean section (Ziegler et al. 1985). This woman lost a significant amount of blood due to the operation, and she was given a transfusion to compensate for blood loss. After six weeks she realised that herself and the infant whom was breastfeeding at the time were both HIV positive. After a major investigation as to how this occurred, it was revealed that the unit of blood she was transfused with was contaminated with the virus (Ziegler et al. 1985).

1.6 Lack of Transmission In The Oral Cavity

Although viral RNA and proviral DNA has been detected in saliva, its transmission via the oral route is rare. This rarity suggests that either the fragments of the virus present in saliva are unable to produce an infection or saliva contains some macromolecules that can inhibit the replication of this virus *in-vitro* (Fultz 1986; Arrietal et al. 2007). Furthermore, the oral cavity consists of epithelial cells which do not express CCR5 or CXCR4 co-receptors making the oral cavity less prone to HIV infection (Millman and Sharma 1994). However, Lamm (1997) demonstrated that epithelial cells from humans could be infected by HIV *in vitro* and that once this infection is transmitted to neighbouring leukocytes, secretory immunoglobulin A (slgA)neutralises it. Immunoglobulins such as IgM and IgG have also been shown to inhibit the transcytosis of HIV via epithelial cells in the oral cavity (Bomsel et al. 1998; Hocini and Bomsel 1999). Histological studies have also demonstrated that epithelial cells are indeed infected by HIV therefore elucidating the impression that the absence of infection in the oral cavity is due to presence of non-infectious viral particles.

Researchers then proposed that the virus particles may be viable but unable to infect the receptive cells (Baron et al. 1999; Philips et al. 1994). Fox et al. (1988) showed that in the presence of viable RNA particles, the hypotonic nature of saliva caused lysis of virus and prevented the attachment of those fragments to any cells that were present in the

oral cavity (Fox et al. 1988). Archibald et al. (1990) then demonstrated that whole saliva except parotid saliva played a role in preventing HIV replication and subsequently the infection of targeted cells. These findings were further supported by Bergey et al. (1990) ; Miller et al. (2005) ; Habte et al (2006 ; 2008) suggesting that saliva is indeed inhibitory.

1.7 The Production and Secretion Of Human Saliva

Human Saliva consist of dilute secretions which are produced by various salivary glands. These salivary glands include the parotid, the sublingual and the submandibular and they all have a similar anatomical structure which includes secretory end pieces, acini and an arborized ductal structure that opens into the oral cavity (Holmberg and Hoffman 2014).

Various salivary glands aid in the production and secretion of saliva. The parotid, the submandibular and the sublingual glands are the major salivary glands in the oral cavity. The type of saliva produced in each gland is dependent on the amount of acinar cells present (Bikker et al. 2004). The parotid gland comprises of serous acinar cells which result in the production of watery serous saliva (Amino et al. 2012). This is however different to the submandibular and the sublingual glands which consist of both mucous and serous acinar cells. The mucous cells of these glands secrete an appreciable amount of mucins and glycoconjugates which gives saliva its visco-elastic properties (Holmberg and Hoffman 2014). Saliva also consist of a number of proteins whose function is crucial in the protection of the oral cavity. The different proteins which are found in saliva each have a different and unique role. The role of saliva is most evident in patients that suffer from hyposalivation, a condition in which the secretion of saliva is low. Most of these patients suffer from microbial colonization of oral tissue and opportunistic infections such as *Candida albicans* and *Streptococcus mutans* giving saliva the ability to protect the oral cavity from the colonization by microorganisms (Fox et al. 1985). Saliva is initially isotonic when it is made in the acinar cells but as it passes through the duct systems, the composition of electrolytes is modified by the reabsorption of sodium and the secretion of potassium and bicarbonate ions making saliva hypotonic (Humphrey and Williamson 2001). This hypotonicity allows for the expansion and hydration of mucin glycoproteins and also allows taste buds to perceive the different tastes.

The parasympathetic and the sympathetic branches of the autonomic nervous system stimulates the secretion of saliva (Kahle et al. 2003). The amount of saliva produced by each gland depends on the extent of gland stimulation and during resting conditions, 0.3mL/min is released (Wang et al.1998). This value differs among different individuals. The flow rate of saliva during sleep is approximately zero. During the resting conditions, the amount of saliva produced by the submandibular gland's accounts for 65% of the 0.3mL/min while the parotid gland and the sublingual accounts for 20% and 15% respectively (Humphrey and Williamson 2001). During resting conditions, any flow rate below 0.1mL/min is considered hypofunction (Wang et al. 1998). During stimulation, the flow rate increases to 1.9mL/min and the relative contribution of each gland varies significantly (Wang et al. 1998).

1.8 Human Saliva Composition and Function

The composition and flow rate of saliva differ from one individual to another as a result of the fluctuations in secretions made in different salivary glands. Saliva consists of a variety of electrolytes such as sodium, magnesium , calcium and potassium (Humphrey and Williamson 2001). About 99% of the salivary fluid secretion is water. In addition to that, proteins, enzymes, mucosal glycoproteins together with other antimicrobial factors form the core components of saliva (De Almeida et al. 2008). Although the concentration of glucose, urea and ammonia is minimal, they also form part of the complex components of saliva. The combination of these factors gives saliva its multifunctional characteristics such as digestion, buffering effect, lubrication, remineralisation and the anti-bacterial and anti-fungal properties (Levine et al. 1993).

1.8.1 Antimicrobial Properties of Saliva

Several antibodies and non-immunological factors play a role in the antimicrobial defence of saliva. Secretory immunoglobulin A (slgA) is the most common antibody that plays a significant antimicrobial role in all mucosal linings. It prevents the direct binding of the oral cavity pathogens to the mucosal and pellicle surfaces. It also aids in the agglutination of pathogens by creating cross links thereby promoting clearance from the oral cavity (Marcotte and Lovoie 1998). Furthermore, slgA inhibits the synthesis of bacteria in the oral cavity by decreasing the production of siderophores, a molecule which scavengers iron from lactoferrin as a result inhibiting the proliferation and production of bacteria (Miethke and Marahiel 2007). IgA play a vital role in inhibiting adherence between bacteria and the oral mucosal surfaces (Schenkels et al. 1995). In addition, slgA has been shown to block bacterial interactions with complementary surface receptors by binding to adhesins which reduce the surface charge of and hydrophobicity of bacteria leading to steric hindrance with the host surface receptors (Marcotte and Lovoie 1998). IgM and IgG are found in low concentrations as compared to IgA. These antibodies perform similar antimicrobial functions (Gronblad 1982). However, a hereditary lack of IgA has been shown to cause an increase in the susceptibility of oral diseases questioning the clinical relevance of IgA (Nikfarjam et al. 2004).

On the other hand, saliva contains other non-immunological salivary proteins such as lysozymes, lactoferrin, histatins, cystatins, proline rich proteins (PRP), peroxidases, salivary agglutinin (SAG) and mucin glycoproteins which play a vital role in protecting the oral cavity either by preventing the adhesion of the pathogen to the oral mucosa or neutralising the pathogen. The role these proteins play protecting the oral cavity is explained below.

Lysozyme is a cationic hydrolase which is found in salivary glands, leukocytes and also in gingival crevicular fluid (Dawes et al. 2015). The cationic nature of this enzyme activates endogenous bacterial autolysins which disrupts the integrity of the cell wall which subsequently destroys them. Another mode of action of this enzyme is that it hydrolyses

of the **β** (1-4) bond that is found in the peptidoglycan layer of bacterial cell walls between N-acetylmuramic acid and the N-acetylglucosamine which inhibits bacterial adherence (Laible and Germaine 1985; Dawes et al. 2015).

Several serous cells in saliva secrete lactoferrin which has a broad range of antimicrobial activities. This enzyme is a scavenger for iron and aids in the process of phagocytosis by marking antigen for immune response and in bacterial clearance (Jenssen and Hancock 2009). The removal of iron in the oral cavity causes harm on the survival of many microorganisms that require iron for their metabolic activities such as Streptococcus mutants (Bullen, Rogers and Griffith 1978; Weinberg 1978; Dawes et al. 2015). Furthermore, the surface area of lactoferrin consists of large cationic charges which bind to the cell membrane of gram-negative bacteria resulting in an increase in the cell wall permeability that ultimately causes bacterial lysis (Jenssen and Backvoll 1998).

The parotid and the submandibular glands produce peroxidase or sialo-peroxidase which offers antimicrobial activities by oxidizing thiocyanate in the presence of hydrogen peroxide to hypothiocyanite which is a potent antibacterial substance (Tenovuo et al. 1994). As a result of this process, the cells and proteins are protected from the corrosive effects of hydrogen peroxide in the oral cavity (Edgar 1992; Humphrey and Williamson 2001; Amerongen 2002).

Histatins are also secreted by the parotid and the submandibular glands and they offer antimicrobial activity against some strains of bacteria that are found in the oral cavity by acting as potent inhibitors of growth and development such as in the case of *Candida albicans* (Amerongen et al. 2002). In addition to this, the histidine rich peptides also inhibit hemagglutination of perio pathogens in the cavity mainly Porphyromonas gingivalis (Murakami et al. 1992). Three main types of histatins namely; histatin 1, 3 and 5 are found in saliva and are all derivatives of histatin 3 (Opeinheim et al. 1998). This protein is also known for its anti-inflammatory properties by releasing histamines which targets fungal mitochondria (Helmerhost et al. 1994).

Secretory leukocyte protease inhibitor (SPLI) is a serine protein inhibitor that also contributes to the defense of the oral cavity. Apart from its antiviral properties, SPLI has antifungal and antibacterial properties (Amerongen et al. 2002; Dawes et al. 2015). The cationic nature of this inhibitor allows it to bind to bacterial mRNA and DNA which interferes with the translation process thereby inhibiting the synthesis of certain bacterial strains in particular Escherichia coli (Miller et al. 1987).

Salivary Agglutinin (SAG) together with mucins particularly MUC5B and MUC7, which are heavily glycosylated proteins, contribute to the oral innate system via agglutination and aggregation (Amerongen et al. 2002). This process aids in the removal of pathogens by preventing them to bind to the oral mucosa thereby ensuring there is maximal clearance of the microorganism (Fabian et al. 2015). Often these heavily glycosylated proteins are associated with other salivary proteins such as sIgA to initiate these antimicrobial activities. The inhibition and clearance of bacteria by MUC5B and MUC7 in the oral cavity is well documented in literature. The inhibitory potential of these heavily glycosylated mucins will be discussed further in section two.

1.9. Inhibition Of HIV-1 by Human Saliva

The first study that showed evidence of the anti-HIV-1 activity of saliva *in vitro* was demonstrated by Fultz in 1986. His resulted were further supported by the findings of Fox et al. in 1990. Ever since then, researchers have conducted many studies in an attempt to determine the key components that gives saliva this inhibitory potential. Interestingly, while saliva inhibited the activity of HIV-1 in vitro, little to no inhibitory activity was demonstrated for herpes simplex virus 1 (HSV-1), adenovirus, HIV-2 and simian immunodeficiency virus (SIV) (Malamud et al. 1993; Malamud et al. 1997).

Even though the anti-HIV activity of whole saliva and other glandular secretions primarily the submandibular has been well documented and reported in literature, there

is still some inconsistencies as to which secretion is the most effective at inhibiting HIV-1. (Fox et al. 1988; Archibald and Cole 1990; Malamud et al. 1993; Bergey et al. 1994; Kazmi et al. 2006). At the same time, less inhibition is shown from saliva secreted by the parotid gland (Bergey et al. 1993; Malamud et al. 1993; Kazmi et al. 2006). Most evidence of the inhibitory potential of saliva has been attributed to the isotonic nature of whole saliva, the presence of immunological factors that defend against virus and non-immunological proteins of the innate cavity which provide antimicrobial defense (Matsuda et al. 1993; Artenstein et al. 1997; Yasuda et al. 1998).

Cell to cell transmission of HIV-1 across the mucosal surfaces has been suggested to be more efficient as compared to the transmission of cell-free virus (Kolodkin-Gal et al. 2013). The presence of non-proviral DNA in saliva was demonstrated by Baron et al. (1991). These findings were supported by Baron et al. (1999) who demonstrated that the hypotonicity of saliva resulted in the lysis of infected leukocytes by osmosis subsequently affecting the replication cycle. Furthermore, when these effects were reversed by the addition of concentrated salts to saliva, no lysis of cells occurred clearly indicating the function of hypotonic saliva (Baron, Poast, and Cloyd 1999).

A similar study was conducted on breast milk to investigate the effect of its isotonic nature on infected cells. This study demonstrated that breast milk is diluted by saliva which changes its tonicity which prevents the disruption of infected leukocytes (Baron et al. 2000). This observation could be the reason why there is variable HIV transmission during breastfeeding. Experiments that were conducted on physiological semen also showed the same observations. According to Baron et al. (2002), during oral sex, the amount of HIV found in semen is sufficient to cause an infection but the hypotonicity of saliva inhibits the infection process allowing the viral particles to cross the oral cavity. However, these results contradict what we know about the rare nature of transmission of HIV-1 via the oral cavity (Romero et al. 2002; Page-Shafer et al. 2002). In addition to this, the oral mucosa is susceptible to cell free virus which is not disrupted by the hypotonicity of saliva (Moore et al. 2003). Therefore this suggests that there are other mechanisms of inhibition that are provided by saliva.

Salivary antibodies namely SlgA and SlgM are known to play a role in the inhibition of HIV-1. These antibodies are highly detected in HIV positive individuals (Matsuda et al. 1993; Artenstein et al. 1997; Yasuda et al. 1998). Furthermore, studies have shown that individuals that are exposed but not infected with HIV have detectable levels of active HIV-1 specific antibodies (Devito et al. 2000; Broliden et al. 2001; Farquhar et al. 2008). These salivary antibodies have been shown to inhibit HIV-1 *in vitro.* (Cartry et al. 1997; Moja et al. 2000). IgA is one of the major mucosal antibody with the highest concentration in the parotid secretions. It shows a high reactivity against the pol and env products of HIV-1 (Cartry et al. 1997; Moja et al. 2000). The inhibition of HIV-1 by saliva from both positive and negative individuals provides proof that HIV specific antibodies cannot solely be responsible for the inhibition of saliva against HIV (Fox et al. 1989).

Although HIV-1 infection elicit an immune response, there is still a lot of evidence that demonstrates the role of non-immunological factors in the inhibition of HIV-1. Mucins, SAG, SPL1, defensins and lactoferrin are the major salivary proteins that have been implicated in the anti-HIV-1 activity of human saliva. The degree to which these proteins contribute towards this inhibitory activity is debatable.

A number of electron microscopy studies and filtration experiments have shown that the inhibition of the HIV-1 particles by saliva is via aggregation or agglutination and this has become the proposed mechanism through which viral inhibition occurs (Archibald and Cole 1990; Yeh et al. 1992; Malamud et al. 1993; Bergey et al. 1993). The incubation of whole saliva and secretions from the submandibular glands with HIV-1 particles, followed by a passage through a 0.45μm filter showed a reduction in the transmission of the virus (Archibald and Cole 1990; Yeh et al. 1992; Malamud et al. 1993). This reduction in viral activity was proposed to be caused by high molecular weight mucin glycoproteins in saliva (Bergey et al. 1994). This postulation was supported by Malamud (1993), when he demonstrated that saliva from the parotid gland, which is devoid of mucins showed no interaction or activity with HIV-1 particles. Moreover, the highest antiviral activity was detected from the secretions of the sublingual and the submandibular glands (Malamud

et al. 1993). Further studies also demonstrated that there is a strong association which occurs between the salivary mucin, MUC7 and the submandibular protein, SAG which results in the formation of a complex with the HIV-1 env protein gp120 virus, causing the dissociation of gp120 from the virus (Nagashunmugam et al. 1998). Therefore, it has been hypothesized that salivary glycoproteins inhibit viral infection and replication by aggregation of viral particles or by shedding of the viral envelope. (Nagashunmugam et al. 1998).

In-vitro studies have confirmed MUC5B and MUC7 as the major salivary glycoproteins that inhibits HIV-1 transmission. In addition to this, crude and purified breastmilk, pregnant mucus plugs together with other purified mucins from other parts of the body have also been demonstrated to inhibit viral replication (Habte et al. 2006; Habte, de Beer, Lotz, Tyler, Schoeman, et al. 2008; Mthembu et al. 2014).

Yeh et al. (1992) noted that after the filtration of whole saliva with HIV particles, there still remained intact HIV-1 particles in the filtrate that were sufficient to cause viral infection (Yeh et al. 1992). This experiment provided a possibility that other small filtrable soluble components played an important role in the inhibition of HIV-1. One such factor is SLPI, a cationic serine protein which has a molecular weight of 11.7kDa. Its anti-activity was first demonstrated in monocytes and CD4 cells at physiological concentration. This protein interacts with the host cells preventing viral binding and subsequent viral infection to occur (McNeely et al. 1995; McNeely et al. 1997; Shugars, Sauls, and Weinberg 1997; Ma et al. 2004). SLPI is also a target to annexin 11, a cofactor protein for macrophage HIV-1 infection (Ma et al. 2004). Contrary to this finding, a study by Turpin et al. (1996) showed no inhibitory activity of HIV-1 by SLPI when tested under different conditions such as the use of different strains of HIV-1, using different target cell lines (Turpin et al. 1996). These results were further supported by findings of Konopka et al. (1999) who suggested that the variable effects shown by SLPI when it came to viral replication and infection was because of the differential expression of cell surface molecules on the target cells. These surface molecules could either allow SLPI to

bind to host cells and hinder host cell infection or they could cause steric hindrance.

In an attempt to elucidate the role of SLPI, Kazmi et al. (2006) demonstrated that in addition to SLPI, two other salivary proteins of different molecular weight sizes displayed inhibition against HIV-1. These results revealed lactoferrin, an 80 kDa protein as the most potent, followed by SLPI and MUC7 (Kazmi et al. 2006). He then proposed that these salivary components affect the HIV-1 cycle at different stages hence the difference in potency. This postulation was in agreement with the findings of Bolscher et al. (2002) when he showed that inhibition can occur before and after HIV-1 replication (Bolscher et al. 2002).

The two different forms of lactoferrin, human and bovine have been shown to inhibit HIV-1 in vitro (Harmsen et al. 1995; Berkhout et al. 2002; Berkhout et al. 2004). Lactoferrin has been shown to inhibit the HIV-1 infection cycle enzyme, reverse transcriptase (Ng et al. 2001). Furthermore, it has been shown to cause viral shedding of HIV-1 particles by binding to the V3 domain of HIV-1 gp120 (Swart et al. 1998) and blocks viral transmission to host cells by binding to the dendritic cell-specific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (Groot et al. 2005).

An agreement as to which salivary factor contribute the most in providing defense against microbial colonization in the oral cavity is still yet to be reached. It is still very difficult to compare which salivary protein has the greatest potency due to the variation that exists between the viral strains, the type of target cells used, experimental conditions and the benchmark that is used to determine inhibition. In spite of that, the experimental results and hypothesis postulated all provide evidence that saliva contains components which provide a synergistic effect (Yeh et al. 1992; Bolscher et al. 2002; Kazmi et al. 2006).

CHAPTER TWO: THE INHIBITION OF HIV-1 BY SALIVARY MUC5B AND MUC7 FROM SELF DECLARED HIV-NEGATIVE INDIVIDUALS.

GENERAL INTRODUCTION

2.1 Mucus

The epithelial surfaces of the respiratory tract, the reproductive tract, the auditory system, the gastrointestinal tract, the oral cavity and the lacrimal glands are protected from dehydration by a viscoelastic gel secreted by the mucosa (Allen 1978; Perez-Vilar & Mabolo 2007). This mucus gel is composed of water, mucus secretions, ions, proteins and lipids together with mucous glycoproteins but the composition of these molecules vary in some pathophysiological cases (Creeth 1978; Rachagani et al. 2009). The main function of this crude mucus gel is to act as a lubricant and provide a barrier against the external hostile environment of the lumen (Allen 1978; Sellers et al. 1988). Its localisation is usually associated with the specific function of the organ. For example, in the stomach the mucus secreted protects the epithelial lining of the stomach from the mechanical force and abrasive effects of hydrochloric acid used in digestion (Allen 1981). In the respiratory tract, foreign particles are trapped by this mucus gel and are subsequently removed with the help of the ciliary action (Fahy et al. 2010; Mall et al. 2017). In addition, the mucus produced in the vagina protects the epithelium during sexual intercourse and during menstruation, the cervical mucus plug thickness change in anticipation of fertilisation (Allen 1981; Carlstedt et al. 1983). The epithelial surface of the colon is protected by a mucus layer from bacteria and the hard faecal material. This muco-adhesive layer also acts as a lubricant against the detergent effects of bile and other surface active chemicals in the epithelium of the gallbladder (Van der Sluis et al. 2006; Johansson et al. 2008; Mall et al. 2017).

2.2 Mucins and Their Types

Mucins are high molecular weight, heavily glycosylated proteins which form the core components of mucus gels (Forstner et al. 1994; Corfield et al. 2001). They provide the

rheological and physicochemical properties of crude mucus which gives them the ability to form gels and aid in their viscosity (Allen 1978; Sellers et al. 1988). The protein cores of mucins (apo mucins) are encoded by mucin genes and they undergo extensive post translational modification which results in the heavily glycosylated nature of mucins (Zalewska et al. 2000). The amino acid composition and chromosomal location of mucins divides them into structurally and functionally distinct groups (Sasaki et al. 2007). Currently, mucins are divided into three categories namely; the membrane bound mucins, the secreted gel forming mucins and the non-gel forming mucins also known as the soluble mucins (Rose and Voynow 2006; Corfield 2015). MUC2, MUC5AC, MUC5B, MUC6 and MUC19 are secreted gel forming mucins and they have cysteine rich domains whilst MUC7, MUC8 and MUC9 are the non-gel forming mucins (Rose & Voynow 2006; Rousseau et al. 2008; Rachagani et al. 2009; Zalewska et al. 2000). The membrane-bound mucins include MUC1, MUC3, MUC3B, MUC4, MUC11, MUC12, MUC13, MUC16, MUC17, MUC20 together with MUC21 (Rose and Voynow 2006; Mall 2008 Corfield 2015; Frenkel et al. 2015).These mucins are important for cell to cell signal transduction and for cell to extracellular matrix interactions due to their association with cytosolic and cytoskeletal proteins (Rachagani et al. 2009; Jonckheere et al. 2010 ; Mall et al. 2017). Transmembrane mucins also play a role in the biological properties of cancerous cells. For example, MUC16 is used as a diagnostic marker for ovarian cancer because of its highly elevated levels in patients that suffer from this disease (Jonckheere et al. 2010; Marcos-Silva et al. 2014; Das et al. 2015; Mall et al. 2017).

2.3 The Physical and Biochemical Structure Of Mucins

Mucins consists of a protein backbone which is linked to a carbohydrate side chain via an O-linked glycosidic bond to either serine and threonine residues (Yurewicz & Moghissi, 1981). Each mucin subunit consists of areas that are both glycosylated and non-glycosylated. It should be noted that glycosylation of mucins mainly occurs in the protein core region that has variable tandem repeats of serine, proline and threonine residues (Pigman et al. 1973; Allen 1982; Zalewska et al. 2000). The tandem repeats in the protein core of mucins vary considerably and are altered by either O-linked glycans or a few N-linked glycans (Rachagani et al.2009). About 80% of the mucin moiety is composed of oligosaccharides which results in their high molecular weight and also serve to protect

the protein core structure of mucins from proteolytic degradation (Carlstedt & Sheen 1984). The sequence and extent of glycosylation vary depending on the localisation of the mucin (Wu, Sako and Herp 1994).

Mucin oligosaccharides consists primarily of N-acetylgalactosamine (GalNAc), N-acetylglucosamine (GlcNAc), N-acetylneuraminic acid (NeuAc, a sialidase) fucose and galactose (Zalewska et al. 2000 Bansil et al. 2006). The N-acetylgalactosamine (GalNAc) is responsible for linking the carbohydrates side chains to the protein core of mucins via serine and threonine residues (Allen 1981). The proline residue is responsible for keeping the interactions between the carbohydrate side chains tight (Allen 1981). The cysteine residues found in the naked regions of the protein core structure of mucins are joined together by disulphide bonds to form mucin subunits (Carlstedt & Sheehan 1984). These subunits have carbohydrates side chains which repel one another resulting in mucin gel polymers, which upon increase in mucin concentration, increases their viscosity (Sellers et al. 1988). The negative charge found on mucins is due to the presence of sialic acid and fucose (Rose and Voynow 2006).

2.4 Confirmation of Mucin Structure

When it comes to the structure and conformation of mucins, there has been some controversy. Two different models have been hypothesised. Using pig gastric mucus, Allen and co-workers proposed the 'windmill' theory of mucins. He demonstrated that when purified mucins of molecular weight 2 x 10^6 daltons were reduced by disrupting the disulphide bonds, four glycoprotein subunits each weighing 5x10^6 were produced. The C terminal region of these subunits were joined together by disulphide bonds via a low molecular weight interlinking protein (Figure 1). The structure looked like a windmill and this became known as the windmill theory (Allen and Snary 1972; Scawen and Allen 1977; Pearson, Allen, and Parry 1981).

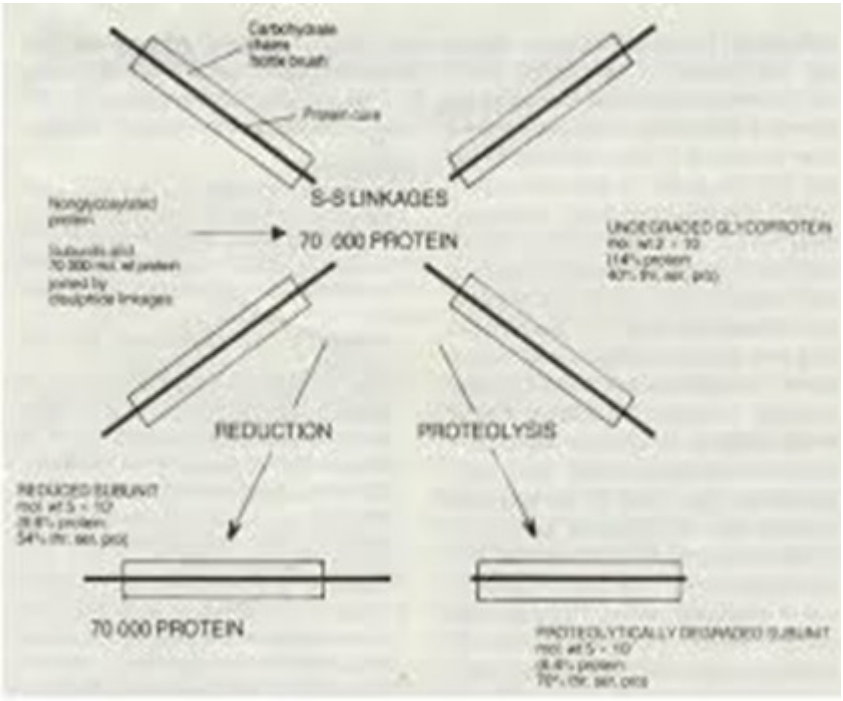


Figure 1 The windmill model of mucin structure as described by Allen et al. (1972).This illustration showed 4 glycoprotein subunits that were joined together by disulphide bonds via an interlinking protein.

A different model in which mucins were joined end to end through disulphide bonds in a linear structure was proposed by Carlstedt et al. (1984). The conformation of these linear bound mucins had naked regions in between the glycosylated regions resulting in a random coil structure (Carlstedt and Sheehan 1984a; Carlstedt and Sheehan 1984b). This random coil theory was supported by the molecular weight and shape sensitive parameters of the mucin glycoprotein (Carlstedt and Sheehan 1984b). The molecular weight observed by Carlstedt group was larger as compared to the one reported by Allen and Snary. Mucins weighed between 10 x 10^6 Dalton and 45 x 10^6 Daltons andupon

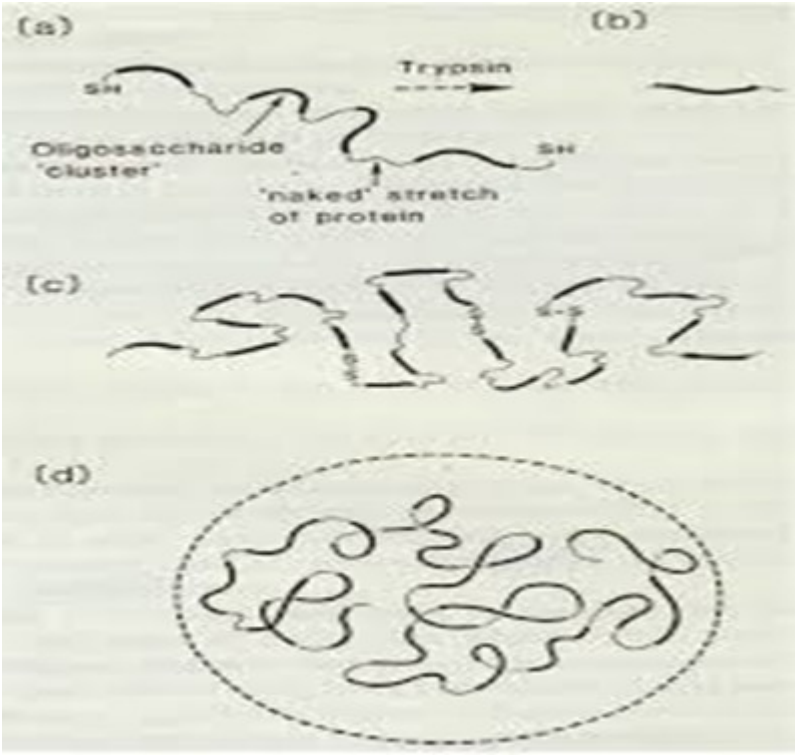


Figure 2 The random coil model of mucin structure proposed by Carlstedt et al. (1984). Mucins joined end to end through disulphide bonds in a randomly coiled manner.

further experiments, the shape sensitive parameters did not change on reduction of these macromolecules(Figure 2) (Carlstedt and Sheehan 1984a; Carlstedt and Sheehan 1984b).

In trying to solve the disparities between the two structures, Mall et al. (1988) attributed these differences to the extraction methods that was used during mucin purification. Allen et al. (1972, 1977, and 1981) used 0.2M sodium chloride (NaCl) as an extraction buffer and did not consider the effects of endogenous proteolysis. It is thought that the absence of protease inhibitors in this medium could have caused mucins to separate into subunits or lead to the proteolytic degradation of the naked regions of mucins subsequently resulting in a smaller size mucin conformation or the windmill model (Scawen and Allen 1977; Allen and Snary 1972; Pearson, Allen, and Parry 1981). On the other hand, Carlstedt and co-workers (1984) used a highly chaotropic medium, Guanidinium chloride (GuHCL), which contained a cocktail of protease inhibitors. These Inhibitors denatured all the proteolytic enzymes and also disrupted the tertiary structure of mucins resulting in the linear model (Carlstedt and Sheehan 1984a; Carlstedt and Sheehan 1984b; Mall et al. 1988). Even though there is still some dispute regarding the

conformation of mucins, the linear model has been proposed to be an accurate description of the conformation of the gel forming mucins (Mall, 1988). GuHCL is still being used as an extraction buffer for mucins because of its ability to keep the integrity of mucins intact and also during purification where it prevents proteins and other impurities from binding to mucin glycoproteins (Thornton et al. 2001). It has been suggested by Mall (1988) that the dialysis removal of GuHCL results in aggregation of mucins.

2.5 Salivary Mucins

Two structurally distinct, carbohydrate rich mucins namely MG1 and MG2 are present in saliva (Thornton et al. 1999). These mucins contribute to the rheological properties of saliva such as viscosity and elasticity and they are secreted by the submandibular and the sublingual glands (Zalewska et al. 2004). On the other hand, the parotid gland is devoid of salivary mucins (Veerman et.al 1996). MUC5B also known as MG1 is a high molecular weight mucin whose function is associated with the encapsulation of a number of microorganisms (Thornton et. al. 1999). This mucin mainly contributes to the viscous and elastic nature of saliva. MUC5B is a heavily glycosylated protein of higher molecular weight, which is encoded by MUC5B gene on chromosome 11, locus p15.5 (Rose & Voynow, 2006). It is one of the widely distributed mucins in saliva, the female genital tract and in the respiratory tract (Argueso et. al, 2002). MUC7 also known as MG2 is a low molecular weight mucin which gives saliva its stickiness (Amerongen et al. 2007). This mucin is located on Chromosome 4q13.q21 and has of approximately 120 000 Da (Rose & Voynow, 2006). Unlike MUC5B, MUC7 is only found in saliva. These two salivary mucins have distinctive domain structure which influence their physical characteristics and their localization in the oral cavity (Linden et. al 1998). Chen et al. (2004) demonstrated the presence of MUC19 in human saliva but no putative peptides have been detected since then (Rousseau et al. 2008).

2.6 MUC5B

MUC5B is one of the largest oligomeric gel forming mucins which has subunits linked to disulphide bonds which are estimated to be between 2.5-2.9x10^6Da each (Thornton et al.

1999). This mucin is primarily secreted by the salivary mucous cells (Bolscher et al. 1995; Nielsen et al. 1997). MUC5B has been detected in the epithelium of the esophageal, the endocervix and in the pancreatobiliary system (Audie et al.1993; Thornton et al. 1999; Vandenhaute et al. 1997).

The gene coding for MUC5B is clustered with MUC2, MUC5AC and MUC6 which are all gel forming mucins on chromosome 11p15.5 (Desseyn, Guyonnet-Dupérat et al. 1997). The apo mucin core of MUC5B is made up of 5000 amino acids which are divided into different domains namely the N-terminal, the central and the C domain. The N-terminal domain has 4 sites where N-linked glycosylation occurs, and this cysteine rich domain consists of 450 amino acids (Cao et al. 2012). The glycosylated structure of MUC5B results in a heavy molecular weight of 14 – 40MDa (Piras et al. 2011).

The central domain is made up of 3750 amino acids which are encoded by an exon and are divided into 19 subdomains (Desseyn, Guyonnet-Dupérat, et al. 1997; Zalewska et al. 2000). The central subdomains contain seven cysteine rich domains named (Cys1-7), three subdomains that have no repeats known as (RO1-RO3), five tandem rich repeats regions that are irregular called (RI-RV) and four uniquely conserved domains which have no repeats known as (RI-end – RIV-end) (Desseyn, Guyonnet-Dupérat, et al. 1997; Zalewska et al. 2000; Cao et al. 2012). These subdomains tend to form composite super repeats which have alternating subdomains of the cysteine rich repeats, one irregular tandem repeat subdomain with 11 repeats of the irregular repeat of 29 amino acid residues, and a unique conserved subdomain with no typical repeats (Zalewska et al. 2000).

The amino acid content of the central domain consists of serine, proline and threonine. Serine constituents 27% of the whole amino acid content while threonine and proline contribute 12.9% and 10.6% respectively (Zalewska et al. 2000). The irregular tandem repeats, RI-end subdomains and subdomains with no repeats are sites that are usually O-glycosylated (Zalewska et al. 2000). The site at which a GalNAc attachment forms has an amino acid sequence known as TXXP (where X is any amino acid) which starts with

threonine followed by any two amino acids and proline. This site also contains many irregular tandem repeats subdomains and most of this sequence is found in the R-end subdomain (Zalewska et al. 2000). There are only seven potential sites of N-glycosylation throughout the whole central domain of MUC5B (Zalewska et al. 2000). The cysteine rich residues found in the central domain are the formation sites of disulphide bridges and it is reported that little glycosylation occurs in this area (Zalewska et al. 2000).

MUC5B structure is referred to as the bottle brush because of the oligosaccharide chains which range between 1-20 residues that are clustered on the super-repeat subdomains. Each monomeric unit consists of approximately 290 O-linked chains that have sialic acids at the non-reducing ends (Thomsson et al. 2002). The presence of different sulphated oligosaccharides and sialic acid side chains on MUC5B has resulted in different glycoforms of MUC5B (Bolscher et al. 1995; Wickström et al. 1998). The C domain of MUC5B contains 808 amino acid residues which have a high proline and cysteine content (Desseyn, Aubert, et al. 1997). The location and number of cysteine residues in the C domain of MUC5B is similar to MUC2, MUC5AC and the on the von Willebrand factor (Troxler et al. 1997; Gum et al. 1992; Meezaman et al. 1994; Titani et al. 1986). In addition, this section is also largely N-glycosylated and contains many naked protein regions which are sites of hydrophobic binding (Cao et al. 2012; Loomis et al. 1987). The gel forming properties of MUC5B are because of its ability to form disulphide bonds with other mucin subunits (Zalewska et al. 2000).

2.6.1 MUC7

MUC7 has a molecular mass that ranges between 150-200 kDa and is smaller as compared to the other salivary mucin MUC5B (Mehrotra, Thornton and Sheehan 1998). The glycosylation sites present in MUC7 are less as compared to MUC5B and so is the length of the oligosaccharide chains (Levine et al. 1987; Thomsson et al. 2002). This non-gel forming mucin is located on chromosome 4q13-2 and is found only in saliva, secreted by the serous cells of the submandibular, sublingual and palatine salivary glands (Bolscher et al. 1999).

Similar to MUC5B, MUC7 has a mid-section domain that which is flanked by an N-terminal and C-terminal domain. The apo mucin core of MUC7 is made of 357 amino acids of which 144 residues together with the only two cysteine residues found in MUC7 make up the N-terminal domain (Zalewska et al. 2000). This domain is a site for both N and O linked glycosylation specifically 4 N-glycosylation sites and 9 O-linked glycosylation sites (Zalewska et al. 2000). The central domain of MUC7 has a high content of proline and serine and is a potential 0-glycosylation site. The O linked glycosylation chain of this domain forms a chain of about 2 to 7 monosaccharides that are held together by disulphide bonds (Reddy, Levine, and Prakobphol 1985; Levine et al. 1987). This domain contains 138 amino acids which compose of 23 tandem repeats residues (Vinall et al. 2000). The oligosaccharide composition of MUC7 consist of NeuAcα2, 3Galβ1, 3GalNAc, Galβ1, 3GalNAc, and Fucα1, 2Galβ1, 3GalNAc (Levine et al. 1987).

No cysteine residues are found in the C-terminal domain of MUC7, but this domain has a high proline content and consequently it's a potential site for both N and O linked glycosylation (Zalewska et al. 2000). The lack of cysteine residues on the C domain of MUC causes MUC7 to form monomers only rather than polymers. This monomeric mucin is non- gel forming, comprises of 74 amino acids and its subunits tend to aggregate due to the presence of cysteine residues found on the N terminal domain that self-associate (Mehrotra, Thornton, and Sheehan 1998). To date, two forms of MUC7 have been

identified and these glycoforms have the same core amino acid sequence but the content of fucose and sialic acids differ (Ramasubbu et al. 1991).

2.7 The Role of Salivary Mucins

Salivary mucins play a number of functions in the oral cavity. They are involved in lubrication, modulation of the oral flora, formation of mucosal coat, and the production of an enamel pellicle (Tabak et al. 1985). MUC5B aids in the formation of this pellicle coat whose function is associated with the protection of the tooth and oral surfaces from acidic challenges (Tabak et al. 1985; Nieuw Amerongen, Oderkerk, and Driessen 1987). The removal of mucins from submandibular and sublingual saliva before the formation of the pellicle coat has shown a 70% reduction in artificial pellicle ability to inhibit demineralisation of dental enamel i.e (dental caries) showing the importance of salivary mucins (Nieuw Amerongen, Oderkerk, and Driessen 1987).

An important function of salivary mucins is the modulation of the oral flora which subsequently control the colonisation of oral microorganisms. A number of mechanisms are involved in the regulation of the oral microflora by MUC5B and MUC7. As discussed in chapter 1, they associate with antimicrobial protein such as SlgA at the mucosal surface where they are concentrated and retained to perform their antimicrobial activity (Frenkel and Ribbeck 2015a; Gibbins et al. 2015). They also exert direct association with oral bacteria. For example, MUC5B is known to decrease the ability of *Streptococcus mutans* to attach to the oral surface and form biofilms by isolation (Frenkel and Ribbeck 2015b). This isolation prevents the formation of oral infections, which are caused by the metabolic by-products of S. mutans (Frenkel and Ribbeck 2015). It has been postulated that salivary mucins protect the oral cavity by aggregation of bacteria, which facilitates their removal during swallowing. In addition, bacteria also bind specifically to different glycans, which facilitates their selective disposal.

Salivary mucins are also known to facilitate oral clearance of microorganisms by inducing bacterial aggregation and by preventing bacterial adherence to the oral cavity (Gibbons and Qureshi 1978; Koop et al. 1990). It has been well documented that MUC7 is the bacterial binding mucin unlike MUC5B. MUC7 has been associated with specific bacterial strains in particular the streptococci strain (Murray et al. 1982; Ligtenberg et al. 1992; Murray et al. 1992). Tabak (1990) demonstrated that saliva that was depleted of MUC7 and not MUC5B could cause agglutination and subsequently abolish the activity of certain *streptococci strains*. Moreover, he demonstrated that sialic acids present on the MUC7 glycans were involved in the binding to bacteria including several *Streptococcus gordonii* and *Streptococcus sanguis strains* (Murray et al. 1992; Levine et al. 1978).

The trisaccharide sequence NeuAcα2, 3Galβ31, 3 GalNAc found on MUC7 is a target site for bacterial binding (Murray et al. 1982). *E. coli* and *Staphylococcus aureus* are non-streptococcal bacteria known to bind to this salivary mucins (Moshier, Reddy, and Scannapieco 1996; Heo et al. 2013). In addition, the N terminal region of MUC7 consists of cationic peptides and histatin 5 peptides that are shown to exert antifungal activity in vitro (Bobek and Situ 2003; Smith and Bobek 2001). Furthermore, Situ et al.(2003) demonstrated that MUC7 had a strong antifungal activity against *Candida albicans* and *Cryptococcus neoformans.* The loss of salivary function has been reported in cases associated with speech impairment, in orodental disease and in dysphagia, which significantly affects the quality of life. This gives more insight on the role of saliva.

2.7.1 Mucins and Anti-HIV Activity

After Fultz (1986) discovered the inhibitory potential of saliva, most researchers conducted numerous experiments to elucidate the main factor that brought about this action. Most evidence implicated salivary mucins as the main constituents in particular MUC5B and MUC7 (Fox et al. 1988; Yeh et al. 1992; Bergey et al. 1993; Malamud et al. 1993). Electron microscopy studies showed the aggregates of HIV particles by the high molecular weight glycoproteins subsequently reduce viral infection (Archibald and Cole 1990; Yeh et al. 1992). This gave a definitive role of the inhibitory effect of

mucins on the transmission of HIV-1. This observation was further supported by the gel filtration studies of Bergery et al. (1993) and Mamalaud (1993) which demonstrated that the mucin rich fractions of the submandibular and sublingual glands showed a higher inhibition as compared to the mucin free parotid secretion. Moreover, a direct association between the mucin rich saliva and the filtered HIV particles, specifically MUC7 was observed and this partially answers the rarity of HIV transmission via the oral route (Bergey et al. 1994). The study by Nagashunmugam et al. (1997) further strengthened this anti-HIV activity concept by showing a higher inhibitory activity in the submandibular secretions than the parotid secretions. He also demonstrated the concepts of inter-individual variation among the secretions that had a higher inhibitory potential. Furthermore, using anion exchange chromatography, he demonstrated that high molecular weight glycoproteins stripped the virus gp120 envelope thereby hindering the virus ability to infect cells (Nagashunmugam et al. 1998).

The Inhibitory properties of purified salivary MUC5B and MUC7 in vivo have been confirmed in our laboratory (Habte et al. 2006; Peacocke et al. 2012). It was further demonstrated that salivary mucins inhibited HIV transmission via aggregation or trapping of the virus (Habte et al. 2006). These findings led to the investigation of other mucin-based secretions (both crude and purified) such as breast milk, human pregnancy plugs, pig saliva and horse saliva. While purified breast milk mucins MUC1 and MUC4 showed inhibition against HIV-1 in vivo (Habte, de Beer, Lotz, Tyler, Kahn, et al. 2008; Mthembu et al. 2014), crude breast milk did not show any anti-HIV activity (Kahn et al. 2008; Mthembu et al. 2014). It was suggested that this observation was a result of fat globules in crude breast milk, which prevented a physical entrapment of the virus, by breast mucins (Mthembu et al. 2014). However, these findings were refuted by Kazmi et al. (2006) who demonstrated high anti-HIV activity in crude breast milk which was comparable to saliva. These findings were also supported by the in-vivo experiments and findings of Wahl et al. (2015). The complexity of this controversy was highlighted by Lyimo et al. (2009) in which he explained that breast milk indeed provided a certain level of protection against cell free HIV but might be insufficient at blocking cell associated infection. Furthermore, when crude breast milk is inoculated by cell free or cell associated virus and heated or pasteurised, the virus is inactivated providing an

explanation as to how crude milk might inhibit HIV activity (Lyimo et al. 2009). Purified human pregnancy plug together with MUC1, MUC2, MUC5AC has been demonstrated to possess the anti-HIV property (Habte, de Beer, Lotz, Tyler, Schoeman, et al. 2008). However, crude pregnancy plug does not have this anti-HIV activity and it was suggested that the mucin concentration is low to elicit an inhibitory activity (Habte et al. 2008).

Despite most electron studies showing viral entrapment by mucins, the exact mechanism through which mucins inhibits HIV has not yet been identified. There has been some evidence which shows interaction between HIV-1 and both whole saliva and glandular salivary secretions. Viral inhibition by submandibular saliva was shown to affect the virus rather than the target cells (Malamud et al. 1997). This inhibition was only specific to HIV-1. No inhibitory activity was detected against HSV-1, HIV-2, adenovirus and SIV (Malamud et al. 1993; Malamud et al. 1997; Nagashunmugam et al. 1997). These findings suggest that mucins offer some degree of interaction specificity.

Dendritic cells have been demonstrated to transfer HIV-1 in vivo to CD4+T lymphocytes cells without eliciting infection (Geijtenbeek et al.2000a). These antigens presenting cells express binding receptors for HIV-1 called dendritic cell-specific intercellular adhesion molecule-3 grabbing non integrin (DC-SIGN) (Van Liempt et al., 2004; Van Liempt et al., 2006; Guo et al., 2004; van Kooyk and Geijtenbeek, 200). On the other hand, O-linked mucins glycans are rich in Lewis X structures known as DC-SIGN epitopes. Saeland et al. (2009) demonstrated the ability of MUC1 from breast milk to inhibit transmission of HIV-1 from dendritic cells to CD4+ target cells by binding to this receptor. He demonstrated that there is a strong association that occurs between mucin Lewis X and the DC-SIGN receptor of dendritic cells that subsequently blocked binding of the viral gp120 to DC-SIGN resulting in the inhibition of DC-SIGN-mediated transmission from dendritic cells to CD4+ T cells (Saeland et al. 2009). It has been demonstrated that bile-salt stimulated lipase (BSSL) from breastmilk and MUC6 in seminal plasma use DC-SIGN to block transfer (Martijn et al.2009). It should be noted that, there is a possibility that genetic mutations could occur in the lewis X content of MUC1 that could affect the level of protection breast

milk has to offer against HIV-1 which could explain the variable transmission of HIV-1 via breastfeeding (Saeland et al. 2009).

It seems as if there are a number of mechanisms through which HIV transmission by mucin glycoproteins occurs. Currently, viral aggregation between the mucin glycans and the viral envelope proteins is the most accepted hypothesis. This hypothesis is supported by the fact that mucins have a high proportion of carbohydrate side chains, the interaction of MUC7 with gp120 envelope protein and lastly the inhibition that is specific to HIV-1 other than the virus (Malamud et al. 1993; Bergey et al. 1994; Wu, Csako, and Herp 1994; Nagashunmugam et al. 1997; Habte et al. 2006). Evidence is emerging that as with variable interaction that is there between MUC5B and MUC7 with bacteria, the inhibitory mechanisms of these mucins might also be different due to their physical and biochemical characteristics.

2.8 Anti-HIV Microbicides

The idea of using vaginal microbicide gels was first thought of in the early 1900s with the main aim of providing women with a tool that they could use to prevent sexually transmitted infections (STIs) and also to protect them against the infection of HIV-1 (Stein, 1990; Voelker, 2006). In addition, this would allow women to have a preventative tool that they could use without their partners' consent especially in relationships where condom use is difficult (Doggett et al. 2015). By the end of 2016, UNAIDS published a report stating that sixteen million women globally, aged between 15 and below were living with HIV. South Africa was residence to about 13 million female adolescents and young woman that were infected with this virus (UNAIDS, 2014). UNAIDS further reported that the main contributor of this infection among women was due to heterosexual transmission (UNAIDS, 2014). This then emphasized the need of a microbicide that women could use to protect themselves.

Microbicides are chemical substances in the form of gels or creams that are applied to the vagina or rectum to reduce the transmission of STIs such as HIV (Fichorova et al. 2001). They work by enhancing the natural vaginal defence flora, maintaining the acidic pH of the vagina, by providing a physical barrier against HIV to target cells and lastly by preventing replication of the virus once they have infected targeted cells(WHO 2009). Vaginal microbicides that were made initially (first generation), were not effective at providing protection against HIV infection and actually induced genital inflammation and subsequent cytokine responses that enhanced HIV-1 infection in women that used them (Fichorova et al. 2001; Mesquita et al. 2009). The chemicals and formulations that were used in early microbicides disrupted the cellular and microbial membranes and altered the pore size of the mucus coating the genital mucosal surfaces subsequently resulting in inflammation and allergic response (Lai et al., 2010a).

Although most candidate microbicides have shown no significant protection towards HIV transmission, Karim et al. (2010, 2012) formulated an antiretroviral microbicide gel that contained a nucleotide reverse transcriptase inhibitor (NTRI) tenofovir (TFV), which provided a 39% protection against heterosexual transmission of HIV (Karim et al. 2010; Karim et al. 2012). Originally formulated as an oral antiretroviral drug (ARV), Tenofovir has shown high reductions in HIV infection during phase III clinical trials along with a good safety profile (Karim et al. 2012). Unfortunately, the specific nature and mode of action of antiretroviral microbicides creates the potential for drug resistance on recurrent use, which is a noteworthy concern in their use as a microbicidal gels. Other microbicides have been shown to increase the risk of HIV acquisition when used frequently by women who are at high risk remaining an option for woman who are at low risk. In addition, some do not prevent against STIs such as chlamydia and gonorrhoea and cannot be used rectally (WHO 2009). This calls for safe, effective and affordable microbicides that can be used without causing side effects or increasing susceptibility to HIV.

2.9. Rationale and Research Question

Our understanding of the specific way through which mucin glycosylation inhibits the transmission of HIV-1 is still superficial. While giving some important insight into the mechanism of HIV inhibition, we hope that this study will determine the minimum peptide chain length and structure of a gel forming mucin that retains the anti-HIV activity. This is the first time that our laboratory is testing crude saliva and purified salivary mucins against a subtype B strain.

By fully understanding how mucins protects the body against HIV-1 entry, we can potentially reduce viral transmission and by doing so, enhance the body's natural innate immune defences. If able to distinguish the key structural variants of mucins which play part in this defence mechanism, we could use this as the core component of a vaginal or rectal microbicide.

2.9.1 Aims and Objectives

- To access the inhibitory effect of salivary mucins (MUC5B and MUC7) and the effect of reduction and trypsin digestion in the inhibition of HIV-1 *in vitro*.
- To ascertain the role of salivary oligosaccharides /mucin glycans that are required for maximal inhibition.
- To determine the minimum peptide chain length of a gel forming mucin that retains the anti-HIV-1activity in *an in-vitro assay*.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Ethics

This study was approved by the University of Cape Town Research and Ethics Committee. (Ethics approval number HREC; REF 078/2010).

3.2 Materials

Guanidinium hydrochloride (GuHCl), caesium choride (CsCl), acrylamide, Sepharose CL-4B, pararosaniline choride, N,N'-methylenebisacrylamide (Bis/Acrylamide), β-mercaptoethanol, Thiazolyl blue trazolium bromide (MTT), N-Ethylmaleimide (NEM), dialysis tubing, Dulbecco's Modified Eagle's Medium (DMEM), Penicillin Streptomycin, diethylaminoethyl (DEAE) dextran , Fetal bovine serum (FBS) Superior, Non-essential amino acids (NEAA) (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) HEPS buffer, Phosphate-buffered saline(PBS) without Ca^{2}+ or Mg^{2+},.Neuraminidase proteomic Grade enzyme α(2→3,6,8,9) was purchased from Sigma-aldrich (St Louis, USA). Phenylmethylsulfonylfluoride (PMSF), ethylenediaminetetraacetic acid (EDTA), isopropanol, L-lysine, glycine, ammoniumpersulphate (AMPS), N,N,N,N,'-tetramethylethylenediamine (TEMED), periodic acid and Tween 20 Detergent were obtained from Merck (Darmstadt, Germany). Tris(hydroxymethyl)-aminomethane (Tris), sodium dodecyl sulfate (SDS), sodium Metabisulphite, bromophenol blue and nitrocellulose membrane were supplied by Kimix Chemical and Lab Suppliers (Cape Town, South Africa). Glycerol and activated charcoal were from BDH Chemicals (London, UK). Quick StartTM Bradford dye was from Bio-Rad Laboratories (Hercules, California, USA). Methyl orange was from Fluka Analytical (Seelze, Germany). The ChemiFast Chemiluminescent Substrate Kit was from SYNGENE (Cambridge, UK). Hydrochloric acid (HCl) was obtained from Radchem Products, Inc (Orland Park, Illinois, USA). Acetic acid was from Associated Chemical Enterprises (Johannesburg, South Africa). Protein Aqua Stain was from Vacutec (Johannesburg, South Africa). Polyclonal rabbit anti-MUC7 primary antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, California

USA). Monoclonal rabbit anti-MUC5B primary antibody was kindly provided by Dallas Swallow (University College London, UK). Polyclonal goat anti-rabbit and rabbit anti-mouse horse radish peroxidase (HRPO) linked secondary antibodies were supplied by Dako Cytomation (Copenhagen, Denmark). Pre-stained Protein Ladder and trypsin-EDTA solution were from Thermo Fisher Scientific (Cape Town, South Africa). Trypan blue was obtained from Celtic Diagnostics (Dublin, Ireland). Bright -Glo Luciferase Assay Substrate and Bright-Glo Luciferase Assay Buffer were from Promega (Madison, Wisconsin, USA) and the Glycosylation kit was purchased from sigma-aldrich (Saint Louise,USA). The cell proliferation kit (MTT) was purchased from Roche (Mannheim, Germany). TZM-bl/JC cells and Du422.1 pseudo virus was provided by the International Centre for Genetic Engineering and Biotechnology (ICGEB) (Cape Town, South Africa**.**

3.3 Collection of Samples

Human Saliva was collected from twenty-five healthy volunteers who declared a risk-free lifestyle. The volunteers had abstained from eating and drinking at least 2 hours prior to sample collection and immediately before collecting the saliva, the participants had to rinse their mouth with water. Saliva was collected at the same time (10 O'clock) from each participant to allow uniformity.

The production of saliva was stimulated by chewing on parafilm and was collected by spitting into 6M Guanidine hydrochloride (GuHCl) buffer containing a cocktail of protease inhibitors (PI) namely 5mM NEM, 1mM PMSF and 10mM EDTA. The ratio of GuHCl to saliva was 1:3. The samples were mixed overnight at 4°C to solubilise the mucins and they were centrifuged at 105 000g for 30 minutes to remove insoluble debris. The Supernatant was collected and stored at -20°C until further purification.

3.4 Purification of Salivary Mucins

3.4.1 Caesium Chloride Ultracentrifugation.

Mucins from saliva were purified and separated from other contaminants (proteins, nucleic acids etc) using Caesium chloride (CsCl) isopycnic density ultracentrifugation. This method separates macromolecules based on their differences in densities. Briefly, CsCl molecules dissociates into heavy Cs^{2+} and Cl^{-} ions and because of their heavy nature, the force of centrifugation pushes them downwards and the same time this is opposed by the force of diffusion which then results in the formation of a shallow density gradient. The lowest density is formed at the top of the tube which gradually increase until the bottom. Molecules within the sample then migrate to where their buoyant density is equal to that of their surrounding gradient. Mucins are therefore separated based on their densities (Creeth et al. ,1970).

Salivary mucins were purified in accordance with the methods described by Creeth and Denborough (1970). Whole saliva supernatant samples were prepared in 4M GuHCl pH 6.5 containing protease inhibitors and were adjusted to a density between 1.39 grams per millilitre (g/ml) and 1.42g/ml (the particle buoyancy of mucins) with solid caesium chloride. 50 ml Beckman tubes were divided into 9, 1-centimetre (cm) fractions. The adjusted samples were transferred and sealed in Beckman tubes which was then followed with density gradient ultracentrifugation at a temperature of 4°C for 48 hours at 105 000 g in a Beckman L45 ultra-centrifuge.

The resultant fractions were fractionated pipetted into 10ml glass tubes using Pasteur pipettes. The densities of each fraction were measured by weighing 1 ml of the respective sample on an analytical balance. The fractions were analysed for glycoprotein presence using the Periodic Acid Schiff (PAS) assay and protein detection was conducted using the Bradford Assay. Fractions correlating with a positive PAS peak and a density of approximately 1.42g/ml (mucin rich fractions) were pooled and subjected to a second round of centrifugation. The resulting gradients were fractionated, their densities were

measured, and glycoprotein and protein content were measured. The results were used to construct a profile highlighting the glycoprotein and protein rich fractions. Mucin rich fractions (4 to 7) were pooled, dialysed against three changes of distilled water at 4°C and freeze dried.

3.4.2 Dialysis of Samples.

In-order to remove salts and small contaminants from the eluted fractions were which could interference in subsequent analytical procedures, dialysis against distilled water was performed.

Briefly, a 25-millimetre (mm) wide nitrocellulose membrane was boiled in a beaker of water containing 1% (w/v) sodium bicarbonate and 1mM EDTA for 2 minutes to soften it. The glycoprotein rich fractions were pooled, sealed in the nitrocellulose tubing and dialyzed against 3 changes of distilled water (dH20) with continuous stirring on a Fried Electric Magnetic Stirrer (Haifa, Israel), at 4°C. Five hours was the minimum time required before each distilled water change.

3.4.3 Freeze Drying.

This method concentrates the fractions by removing water, which preserves the sample in a stable form (Snowman et al. 1988). During this process, pooled mucin fraction from caesium chloride ultra-centrifugation were frozen at -80°C overnight which was followed by lyophilization at 50°C in a vacuum of 0.021 millibars (mbar) for approximately 48 hours or until completely dehydrated on a Freeze Zone 6 Freeze Dry System (Labconco, Kansas City, USA).

3.4.4 Size exclusion chromatography.

Salivary Mucins (MUC5B and MUC7) were purified using a Sepharose CL-4B column for separation. This technique separates biological materials based on their molecular size. The large molecules are excluded from the gel and move quickly down the column, separating from smaller molecules which diffuse into the pores, restricting their progress. Sepharose CL-4B beads have a fractionation range for globular proteins of 60 to 20 000 kDa.

Before the sample was put on the column, oxygen was removed from sepharose CL-4B beads by bubbling with nitrogen gas to prevent air bubbles that could block or impede the flow of the sample. Following this, the beads were packed into an Amicon Wright gel filtration column (30cm x 2cm, bed volume of approximately 94cm) as described by the manufacturer (Sigma-Aldrich). The column was primed by washing the beads with a 4M GuHCl solution for at least two hours to equilibrate the bed before use. Lyophilised mucin samples were dissolved in a 5ml solution of 4M GuHCl that contained protease inhibitors. Approximately 0.1 mg of methyl orange was added to the sample to visualise its movement through the column. The column was connected to a chromatography fraction collector (FC 204 Fraction Collector, Gilson, Inc. USA) that eluted each fraction at 1 ml per tube.

Fractions of l ml were collected per tube and subjected to PAS (Periodic acid Schiff) for glycoprotein and Bradford Assay for protein determination using the method illustrated by Mantle et al. (1978) and Bradford (1977) respectively. The results were used to construct a profile highlighting the glycoprotein and protein rich fractions. PAS positive peaks eluting in the void volume (V_0) and included volume (V_i), previously identified as MUC5B and MUC7, were pooled separately, dialysed against 3 changes of distilled water (dH20) and freeze dried for further analysis.

3.4.5 Periodic acid Schiff's (PAS) assay for glycoprotein detection.

This assay was used to detects the presence of glycoproteins in our samples. The hydroxyl group(-OH) present in glycoprotein is oxidised to an aldehyde (-CHO) in the presence of periodic acid. The decolourised schyffs reagent then reacts with the aldehyde group(-CHO) resulting in a colour change from colourless to purple/red (Thornton et al. 1996).

20µl of each chromatographed sample fraction was pipetted into a 96 well microtiter plate. 100 µl of Periodic solution (7% acetic acid with 50% Periodic acid) was then added to the plate and the reaction was incubated at 37°C for 1 hour. After incubation, 100µl of the decolourised schyffs reagent (0.1g sodium metabisulphite in 6mls schyffs reagents) was added. This was then followed by a further incubation period of 30 minutes at room temperature before the absorbance readings were taken at 585 nm on an Anthos HTII Plate Reader (GoIndustry DoveBid, Lancashire, United Kingdom).

3.4.6 Bradford assay for protein detection.

The Bradford protein assay was used to measure the concentration of total protein in our samples. The principle of this modified assay is that it utilises a protein-binding dye that exists in 3 forms; cationic, neutral and anionic (Compton et al. 1985). Under acidic conditions, binding of the protein molecules via Van der Waals forces and hydrophobic interaction to the Coomassie dye to form the protein-dye complex, causes a colour change from brown to blue. Briefly, 10µl of our fraction sample was plated on a 96 well plate. 200µl of the Bradford reagent was added and the reaction was allowed to stand at room temperature for 5 minutes. Thereafter, the absorbance was read at 585 nm using an Anthos HTII Plate Reader.

3.5 Chemical Treatments of Samples

Both crude and purified salivary samples were treated as described below. Samples were reduced and digested in accordance with the methods described by Mall (1988).

3.5.1 Reduction of disulphide bonds.

Reduction was performed by incubating 15 ml aliquots of crude saliva in 6M GuHCL with 10 mM DL-dithiothreitol (DTT) at 37°C for 5 hours. Thereafter, iodoacetamide (IAA), an alkylating agent which prevents the formation of disulphide bonds was added. The sample tubes were covered with foil paper and were incubated overnight at room temperature in the dark. Samples were then dialysed against 6M GuHCl in preparation for gel filtration.

3.5.2 Digestion of Proteins.

Digestion of proteins was done by adding 0.125% trypsin with EDTA in the ratio of 1:100 to 15 ml aliquots of crude saliva in 6M GuHCL. This was then followed by an incubation at 37°C overnight. Samples were then dialysed against water and freeze dried in preparation for gel filtration. It is important to note that the samples that were undergoing trypsin digestion were not pH controlled so the optimal trypsin treatment may not have occurred.

3.6 Confirmation of Mucin Size and Purity

3.6.1 Gradient (4-20%) sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE).

A 4-20% SDS Page gradient was used to confirm the molecular size and purity of salivary mucins. The gradient gels were made as described by Laemmli et al. (1970). Briefly, a running gel solution was made up of a 4% light solution and a 20% heavy solution. The light solution was made up of 0.8 ml 30% Bis/Acrylamide, 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8, 3.7 ml dH2O, 30 µl 10% ammonium persulphate (AMPS) and 5 µl N, N, N, N'-tetramethylethylenediamine (TEMED) while the 20% heavy solution was made up of 4ml 30% Bis/Acrylamide, 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8, 0.5 ml dH2O, 30 µl 10% AMPS and 5 µl TEMED. Using the same serological glass pipette, equal volumes(2.3mls) of both the 4% light solution and the 20% heavy solution were pipetted. In order to create the 4-20% gradient, a single bubble was allowed to move throughout the solution by letting some air in the pipette. Thereafter, the running gel was poured into the glass plates (biorad) and it was topped up with methanol to promote polymerisation.

Thereafter, the stacking gel was prepared as follows; 0.5 ml Bis/Acrylamide, 2.8 ml spacer buffer, 73 µl AMPS and 5 µl TEMED. After the gradient gel was set, the methanol was removed by tilting the glass plates on a piece of tissue paper and the stacking gel was poured. Immediately the comb was then inserted to create wells. The freeze-dried samples (1mg/100µl) were reconstituted in sample application buffer which contained 2% SDS, 10% glycerol and 0.01% bromophenol blue. The solution was left overnight at room temperature for the samples to fully dissolve. Aliquots of the sample solution (15 ul) were loaded onto the 4-20% gradient gel and subjected to electrophoresis. 5ul the protein pre-stained molecular weight marker was also loaded. The gels were run at 200V, 25mA for 90 mins in tank buffer (0.025M Tris, 0.19 Glycine, 0.1% SDS). The gels were stained for glycoprotein and protein visualisation using PAS and aqua stain (Vacutec) respectively.

3.6.2 PAS staining of 4-20% SDS-PAGE gel.

The Periodic Acid Schiff (PAS) gel staining described by Dubray and Bezard (1982) was used to visualise glycoproteins in the gels. After gel electrophoresis, the gels were placed in 50% ethanol for 30 minutes which was followed by washing with distilled water for

10 minutes. After washing, the gels were then placed in 1% Periodic Acid made up in in 3% Acetic acid for 30 minutes. Thereafter, the gels were washed with distilled water overnight at 4°C. The gels were washed twice in a solution containing 0.1% Sodium Metabisulphite in 10mM hydrochloric acid. Schiff's reagent was then poured over the gel before being placed in the dark for 1 hour. This was followed by another one hour incubation of the gel with 0.1% Sodium Metabisulphite in 10mM hydrochloric acid in the dark (Dubray et al., 1982). The Glycoprotein bands were visualized on the gel as purple/pink bands and the gels were scanned on a Hewlett Packard desktop scanner and saved to a computer.

3.6.3 Protein Staining of 4-20% SDS-PAGE gel.

ln order to visualise the protein bands, we stained the SDS-PAGE gel with Vacutec Acqua Stain Protein Gel for 10 mins at room temperature (as per manufacturer's instruction). Thereafter the gels were scanned on a Hewlett Packard desktop scanner and saved to a computer.

3.7 Confirmation of Mucin Identity

3.7.1 Slot Blot.

We used slot blots to identify the mucins that were present in our V_0 and V_i fractionated samples. Briefly, we diluted 100µg of our fractionated lyophilized mucin samples with 100ul of the sample application buffer (10% v/v glycerol, 0.01% w/v bromophenol blue in 1X TAE). The nitrocellulose membrane was soaked in 4x saline sodium citrate buffer (SSC) (0.6M NaCl, 0.06M sodium citrate, pH 7) and it was treated with poly-L-lysine (1:50 dilution with SSC buffer) to allow mucins to bind to the membrane. This was followed by vacuum blotting for an hour at 40 mBar using a Pharmacia LKB Vacugene XL vacuum blotter (Kalamazoo, United States).

Following the one hour period, the membranes were probed with anti-MUC5B and anti-MUC7 antibodies and to visualise the mucins. The same methods described in the western blot section was used up till the detection point. Instead of using the alkaline phosphatase secondary antibodies to detect the presence of the mucins, we used the ChemiFast Chemiluminescent Substrate kit (Vacutec). Using this detection method, equal amounts of reagent A and reagent B were mixed, and an appropriate amount was added to cover the membrane before being dried by blotting with filter paper. The membrane was then viewed in a Syngene G-box (Syngene, Haryana, India)

3.8 HIV-1 Pseudo virus Neutralization Assay

The anti-HIV activity of MUC5B and MUC7 was tested using a pseudo virus neutralisation assay developed by Wei et al. (2002). This assay was originally designed to measure neutralizing antibodies response during HIV infection. (Montefiori, 2009). This assay was later modified and validated by the Dorfman laboratory (from the International Centre for Genetic Engineering and Biotechnology (ICGEB), UCT) to test mucins against HIV infection. The viral inhibition potential of this assay was determined by measuring the reduction in the expression of the luciferase reporter genes of TZM-bl cells, regulated by HIV Tat gene after a single round of infection with HIV-1 pseudo virus (Montefiori 2009). This assay together with the cytotoxicity experiment for cell viability were conducted in a P2 Laboratory at the ICGEB unit at UCT.

3.8.1 Pseudo virus constructs.

The Du422 HIV-1 env pseudo virus, a molecularly cloned subtype C virus and YU2 a clade B virus were used to test the inhibitory potential of salivary mucins. Although both the viruses are HIV-1 subtypes, the origin and where they were derived from differ.

These pseudo viruses were prepared using the 293T cell line as the packaging cells. The packaging cells were transfected with an envelope plasmid and an inactivated envelope backbone (SG3-Δ env) which contains the entire genome except the Env protein (Figure 3). This co-transfection process only generates pseudo virus particles able to replicate

only once due to the lack of the envelope gene sequence Montefiori (2009) and Sarzotti-Kelsoe et al. (2014).

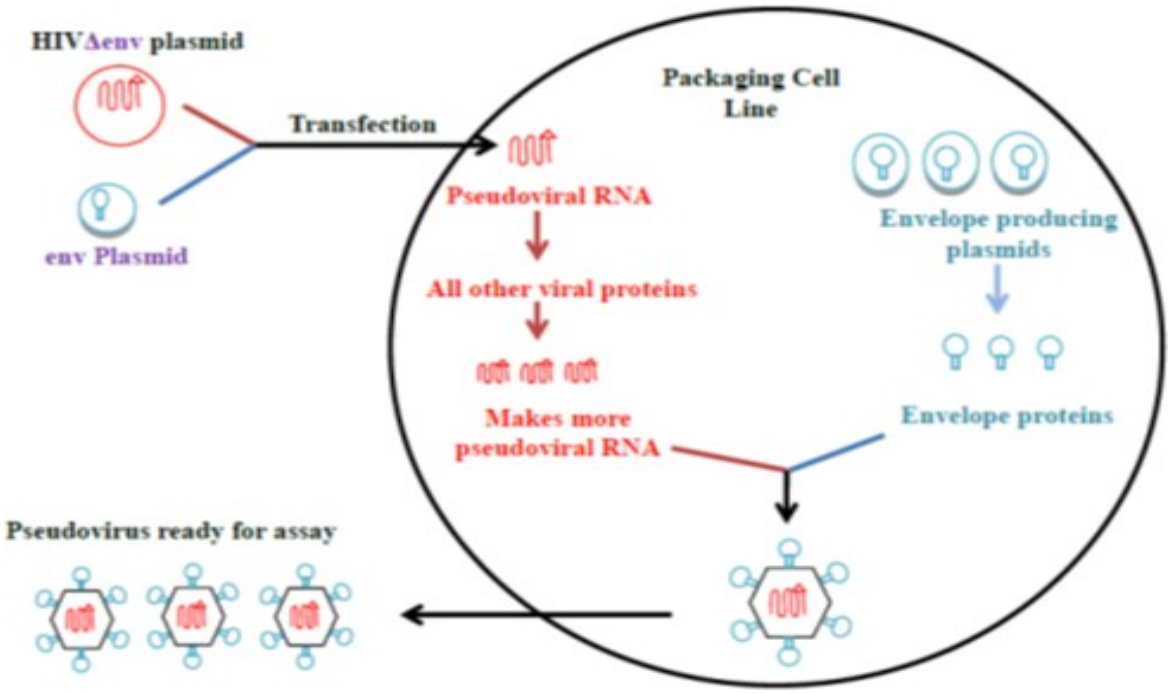


Figure 3 An illustration of how Du422.1 and YU2 pseudo viruses were produced using T293 cells and SG3- Δ env (an inactivated envelope gene) as the plasmid backbone.

3.8.2 Maintenance of target cells.

A Hela cell line, (TZMB/JC) cells which express CCR5 and CXCR4 co- receptors and CD4 receptors was used for both the MTT assay for cell cytotoxicity and the pseudo virus neutralisation assay. These cells are modified with an LTR (long translational repeat) which promotes luciferase and ß-galactosidase gene sequence. (Wei et al. 2002). The virus is made from plasmid backbone SG3*env which transcribes all viral genes except for a dysfunctional (Tat) env gene and a plasmid containing a functional env gene. The functional env is present in different viral strains (i.e. YU2 and DU422). ln-order to determine the viral infection, the HIV-1 Tat gene (from the different virus strains)

initiates the LTR promoter which causes the production of luciferase and ß-galactosidase which is then measured.

TMZB/JC cells were passaged and maintained in 5% Dulbecco's Modified Eagle's Medium (DMEM), containing 5% foetal bovine serum (FBS) and 1% non-essential amino acid (NEAA) 25mM HEPS . These cells were passaged after every 48 hours when the confluency in the plate was between 80-90%. Briefly, 2 mls of trypsin (0.25% trypsin-EDTA) was added to a plate containing confluent cells and this was incubated for 5 minutes at 37°C. Thereafter, the cells were suspended in 8 mls of 5% DMEM and transferred into a falcon tube. The contents of the tube were centrifuged at 1 200 rpm for 5 minutes to pellet the cells. Following this, the supernatant was discarded and 5 mls of fresh 5% DMEM was added to the pellet. The resuspended cells were counted on a haemocytometer.10 µl of resuspended cells were added to 20 µl of trypan blue and 15 µl of trypan blue containing stained cells were counted in three quadrants to obtain the total X x 10^4 cells/ml. One million cells from the suspension were then returned to the plate and 5% DMEM was topped up to make the total volume 13 mls. The cell culture plate was incubated for 48 hours in a 37°C incubator (with 5% CO_2).

3.8.3 Neutralization assay procedure.

The anti-HIV activity of purified treated and crude salivary mucins was tested on TZMB /JC cells using the modified HIV neutralisation assay by Montefiori (2009). Once the cells were confluent as described above, they were resuspended in 10% DMEM, with Diethylamino ethyl-Dextran (DEAE-Dextran) at a concentration of 16.4 mg/ml. The addition of DEAE-Dextran was to remove any charge repulsion that could occur between the virus and the cells. The cells were then adjusted to a density of one million cells per ml and they were plated in a 96 well plate and incubated overnight at 37°C.

On the second day, freeze dried samples of MUC5B, MUC7, crude saliva, trypsin digested saliva, reduced salivary mucins, deglycosylated and desialyated mucins were prepared in 10% DMEM at a concentration of 1mg/ml. The samples (run in triplicates) were serially diluted in half and were plated on a separate 96 well plate (100µl per well). The starting concentration of the DU422 and YU2 was determined using the virus titration method described by Sarzotti-Kelsoe et al. (2014). This method determines the concentration that is required to infect 50% of the cells with the virus. This is also known as the 50 000 relative light units (RLU) and this value was 0.03µl and 0.002µl for DU422 and YU2 respectively. This was the starting concentration used to infect the cells. The virus was then added to the mucin samples and this was incubated for 1 hour at 37°C. Thereafter, the virus-mucin mixture (100µl) was then transfected to the cell culture plate which was plated on day 1 and it was left to incubate for 48 hours. Thereafter, 100 µl of the cell media was removed from each well and 100 µl of Bright-Glo luciferase substrate (Promega, used as per the manufacturer's instructions) was added. After incubating in the dark for 2 minutes at room temperature, 100µl was transferred to a 96-well black plate and luminescence was measured in relative light units (RLU) using a Victor 2 luminometer (Perkin-Elmer). Cells with media served as a negative control while cells with virus but no mucins served as a positive viral control.

3.8.4 Analysis of neutralization data.

In-order for the data to be reliable, the difference between replicates needed to be below 35% of the higher value. To include our samples for analysis, four data points with different concentrations of the same mucin were needed. Viral infectivity was measured by averaging the relative light units (RLU) of the (positive control-negative control). The percentage inhibition was calculated by subtracting viral infectivity from 100. The IC50 defined as the concentration at which 50% of the virus was inhibited by mucins was calculated using the Prism function; variable slope curve fit functions defined as log (inhibitor) vs normalized response (GraphPad, La Jolla, CA, USA). The analysis of data was done using the Mann-Whitney U tests.

3.8.5 MTT assay for cell viability.

The viability of the cells and also the toxicity of our mucin samples, was tested using 3-(4,5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide) (MTT) assay.

This colorimetric assay mimics the neutralisation assay with the exclusion of any virus being used. Viable cells contain mitochondrial enzymes that have the ability to convert the MTT tetrazolium salt into a purple formazan solution. The colour change determines the percentage of viable cells relative to the control cells. This was read on a microplate reader at 595nm.

Briefly, TZM-bl/JC cells (approximately 1 million) were plated in a 96 well plate (10000 cells in each well) and 100µl of 10% DMEM media was added in each well. This was then followed by incubation at 37°C for 24 hours. Concurrently, freeze dried samples (starting concentration 1mg/ml) were dissolved in 10% DMEM media overnight. After 24 hours, mucin samples were serial diluted by half in a different plate done 7 times from the starting concentration of 1mg/ml). Thereafter, 100ul from the cell culture plate was discarded from each well and 100ul of mucin sample from the dilution plate was added. Samples were assayed in triplicates. Cells plated without mucins were used as a positive control, and media was plated as a blank. This was then incubated for 44 hours in a 37°C incubator (5% CO2). Following this incubation period, 10µl MTT (5mg/ml in 10% DMEM) was added to each well and the cell culture plate was incubated for a further 4 hours. Thereafter, 100µl of wells contents were removed and replaced with 100µl of solubilizing buffer (10% SDS in 0.01 M HCl). The plate was incubated overnight at 37°C and after complete solubilization of the purple formazan crystals, the absorbance of the samples was read at 595nm.

3.8.6 Analysis of cell viability data.

In-order for the data to be reliable, the difference between replicates needed to be below 35% of the higher value. To include our samples for analysis, four data points with different concentrations of the same mucin were needed. The percentage viability of the cells was imported to Microsoft Excel and the percentage viability was calculated as the difference between the absorbance of the mucin samples and the media divided by the average difference of the cell control absorbance values and the media. This value was multiplied by 100 to get the percentage. Thereafter, mucins were considered non-toxic if all the cell viability was above 70% and no downward trend in cell viability with increasing mucin concentration was observed. A 50% lethal concentration (LC50) was allocated to samples that had a trend that did not fit the above statement. This (LC50) defined as concentration at which 50% of the cells die was calculated using the log(inhibitor) vs. normalized response -- variable slope curve fit functions in GraphPad Prism (La Jolla, CA, USA).

CHAPTER FOUR: COMPARISON OF THE ANTI-HIV-1 POTENCY OF SALIVARY GLYCOPROTEINS

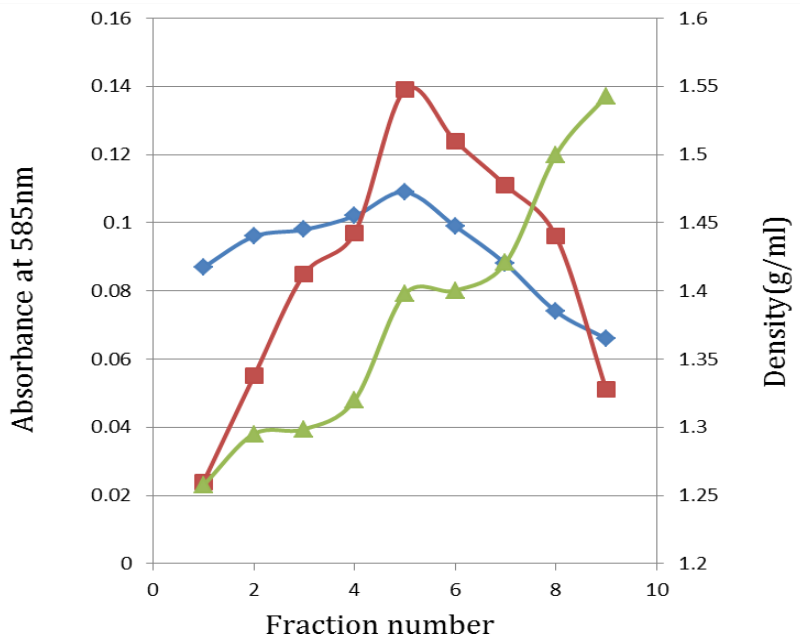
4.1 Purification of Salivary Mucins

Caesium chloride isopycnic density gradient ultracentrifugation

Salivary mucins were separated from nucleic acids and protein contaminants using the caesium chloride isopycnic density gradient ultracentrifugation method of Creeth and Denborough (1970). Thereafter, the tubes were fractionated and the glycoprotein and protein content of each fraction was determined using the PAS and Bradford assay. The corresponding density was also measured (g/ml).

After the first ultra-centrifugation round, a mucin peak detected by the PAS assay was observed from the purification profile starting from fraction 4 to fraction 7 (Figure 4a). This peak correlated with the expected mucin particle buoyant between 1.38g/ml and 1.42g/ml. Fractions 1-3 showed high protein content indicating the comparatively low density of small glycoproteins and non-glycosylated proteins. In addition, there was a clear separation of mucin and protein content after one ultracentrifugation spin. However due to the comparatively high protein content after the first ultracentrifugation spin, mucin rich fractions from the first spin were pooled and further subjected to a second round of caesium chloride ultracentrifugation. Results from the second caesium spin (Figure 4b) showed a low protein peak and a corresponding glycoprotein peak which was thought to be the protein moiety of mucins.

a)



b)

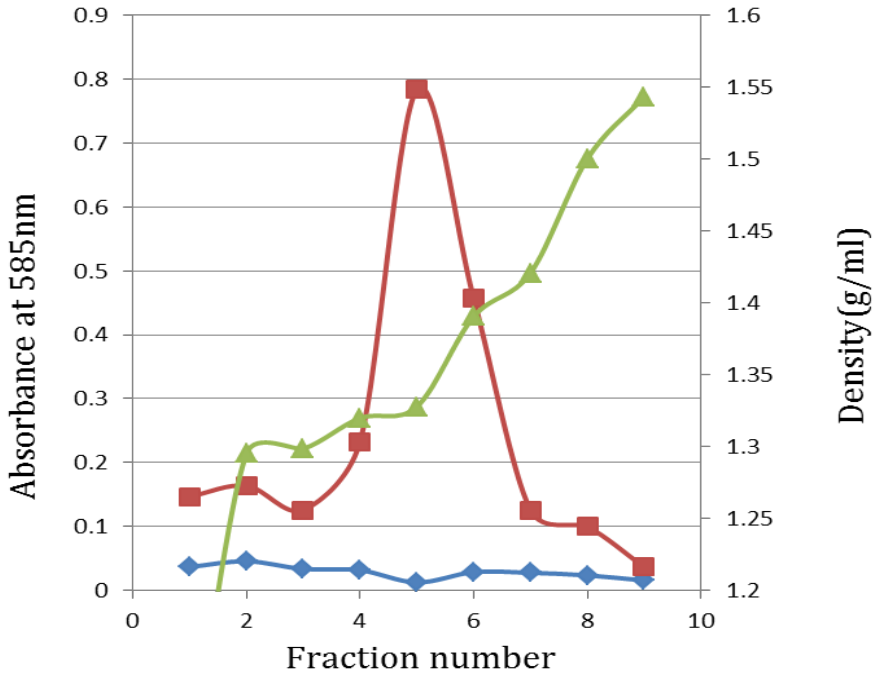


Figure 4 Purification of whole saliva using isopycnic caesium chloride density gradient ultracentrifugation. (a) Shows the gradient profile after the first round of ultracentrifugation and (b) shows the gradient profile following the second round of ultracentrifugation. Red line represents mucin content , blue line represents protein content and green line represents density of mucins.

4.1.2. Gel Filtration

Mucins were separated according to their molecular sizes using the Sepharose CL-4B gel filtration method. The Sepharose CL-4B gel filtration profile (Figure 5) showed two mucin rich macromolecules of different molecular weight sizes. which were detected by the PAS assay . These two glycoproteins were previously identified as MUC5B and MUC7 (Habte et al. 2006). MUC5B eluted first in the void volume (V_o) because of its larger molecular weight size of over 20000kda, whilst MUC7, which is substantially smaller, (150-200kda) eluted in the included volume (V_i) (Figure 5). The V_i and V_o samples were pooled separately, dialysed and freeze dried until needed for further analysis. Salivary mucins from all participants exhibited the same profiles. However, It is worth noting that inter-individual variation was observed in some samples in relation to the sizes of the mucin peaks of MUC5B and MUC7.

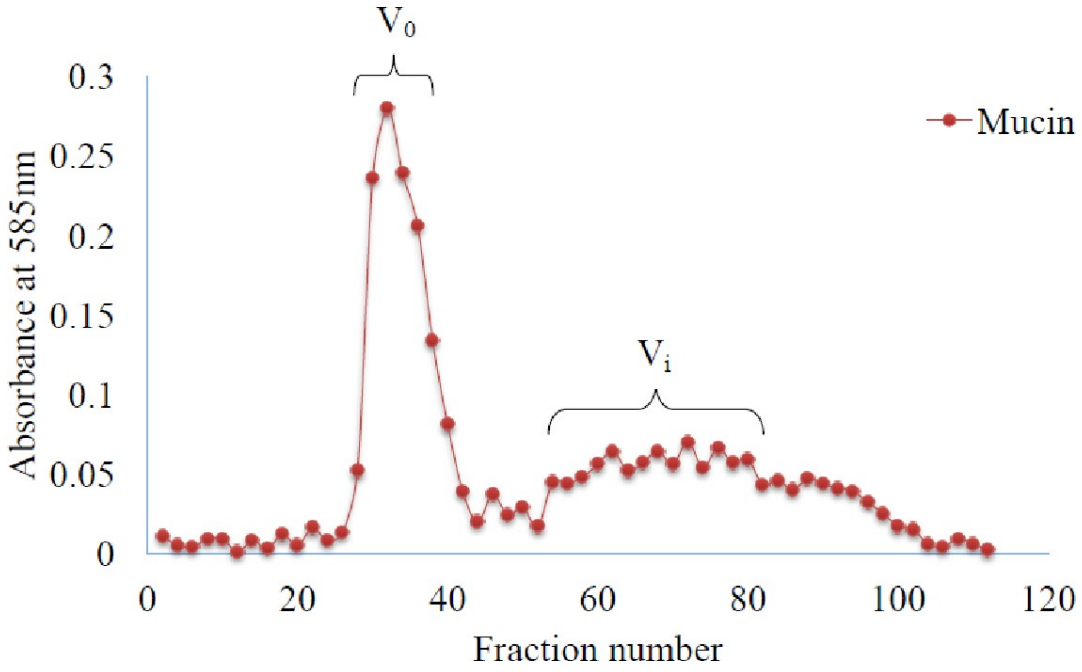


Figure 5 Sepharose CL-4B gel filtration profile of purified saliva. This was used to separated MUC5B and MUC7. The presence of mucins was detected using the PAS assay by testing every second fraction from the column.

4.1.3 Gel Filtration Graphs of Treated Samples

Crude and purified salivary samples that were treated with either DTT or trypsin , were loaded on Sepharose CL-4B gel filtration column in order to monitor the changes in mucin size. Thereafter, the PAS and Bradford assay was conducted to determine the mucin and protein concentration respectively in the eluted material. Allen (1981) reported that proteolytic digestion occurs in the naked regions of the mucin protein core resulting in subunits while the reduction of mucins by DTT breaks the disulphide bonds that join mucin subunits to one another via cysteine residues.

Treatment of crude saliva with 0.25% trypsin yielded an elution profile that had a smaller V_o peak and a larger distinct V_i peak (Figure 6). On the other hand, treatment of crude saliva by DTT followed by alkylation with IAA, yielded a large V_0 peak of undigested mucins and a relatively small V_i peak (Figure 7) suggesting the possibility of a partial digestion on both treatments. Purified trypsin digested mucin samples showed a large population of polymeric mucin which eluted in the V_0, with smaller mucin subunits eluting as a broad and flat peak in the V_i indicating some mucin degradation (Figure 8) Treatment with DTT reduced the size of the V_0 peak, increasing the amount of material eluting in the V_i peak (Figure 9).

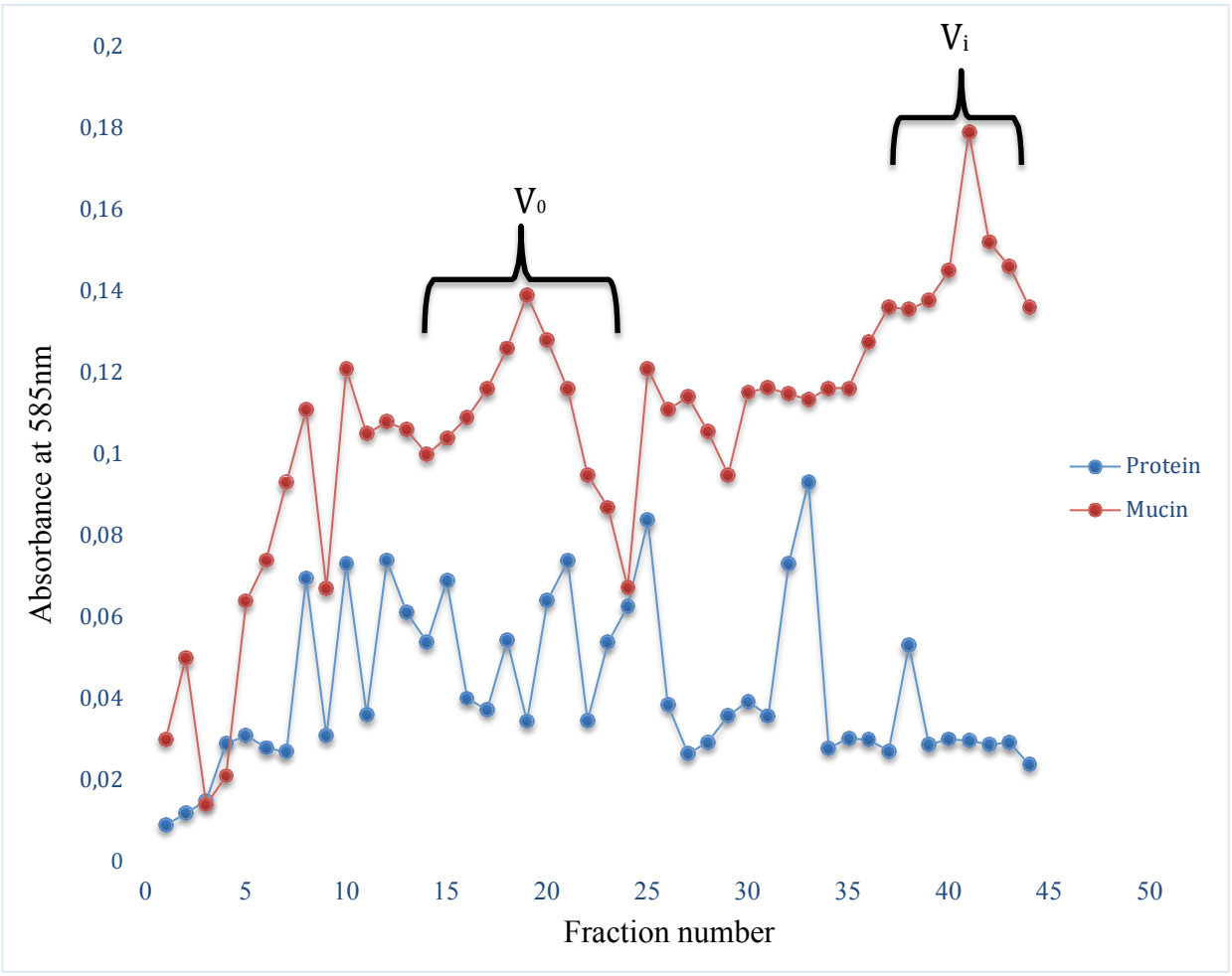


Figure 6 Sepharose CL-4B gel filtration profile of crude saliva that was treated with 0.25% trypsin. Samples were eluted with guanidium chloride buffer through a 40ml bed volume column and l ml fractions were collected, subjected to PAS and Bradford assay to measure the glycoprotein and protein content respectively. Due to the viscous nature of saliva the samples were extracted 1:3 dilution with guanidium chloride buffer.

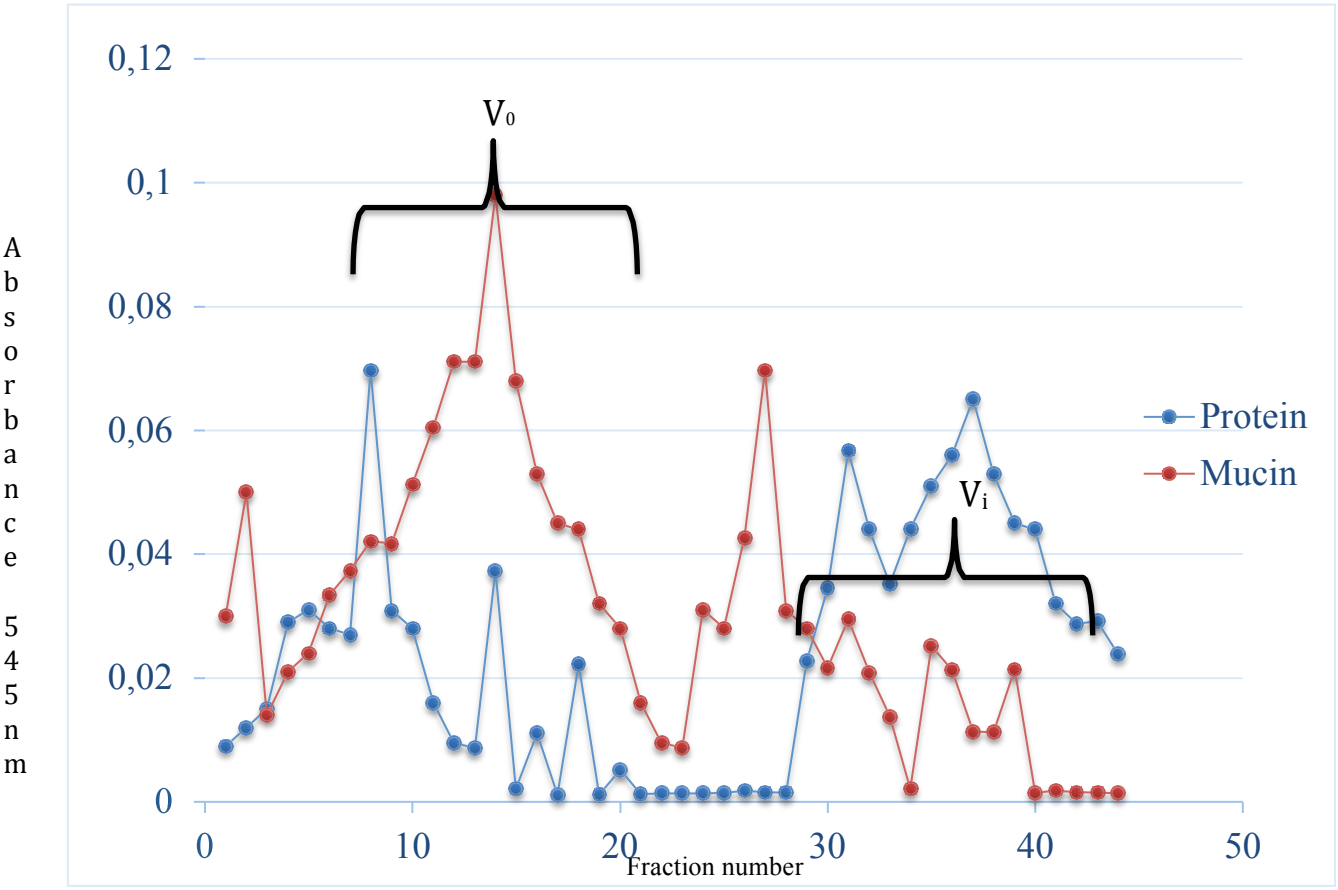


Figure 7 Sepharose CL-4B gel filtration profile of crude saliva that was treated with DTT and alkylated with IAA. . Samples were eluted with guanidium chloride buffer through a 40ml bed volume column and l ml fractions were collected, subjected to PAS and Bradford assay to measure the glycoprotein and protein content respectively. Due to the viscous nature of saliva the samples were extracted in 1:3 dilution with guanidium chloride buffer.

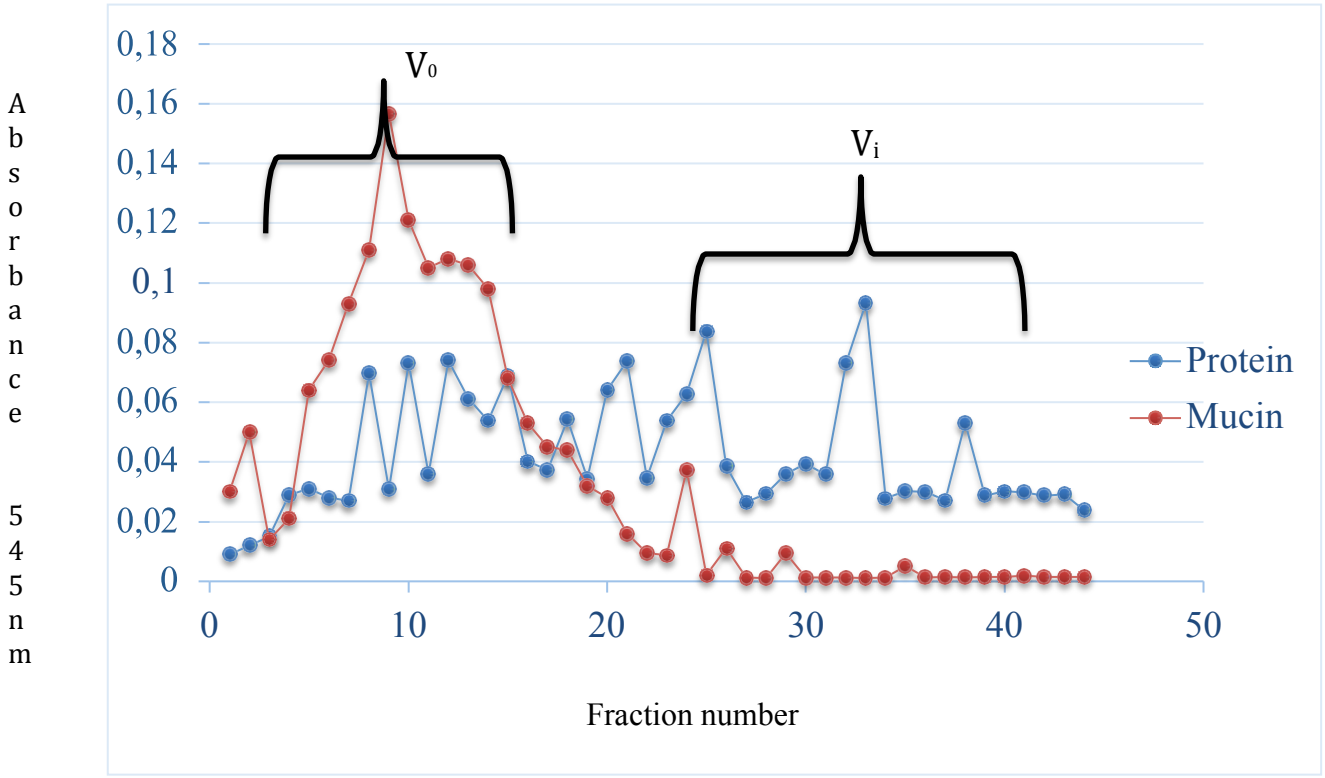


Figure 8 Sepharose CL-4B gel filtration profile of purified salivary mucins treated with 0.25 % Trypsin. Samples were eluted with guanidium chloride buffer through a 40ml bed volume column and l ml fractions were collected, subjected to PAS and Bradford assay to measure the glycoprotein and protein content respectively. Due to the viscous nature of saliva the samples were extracted 1:3 dilution with guanidium chloride buffer

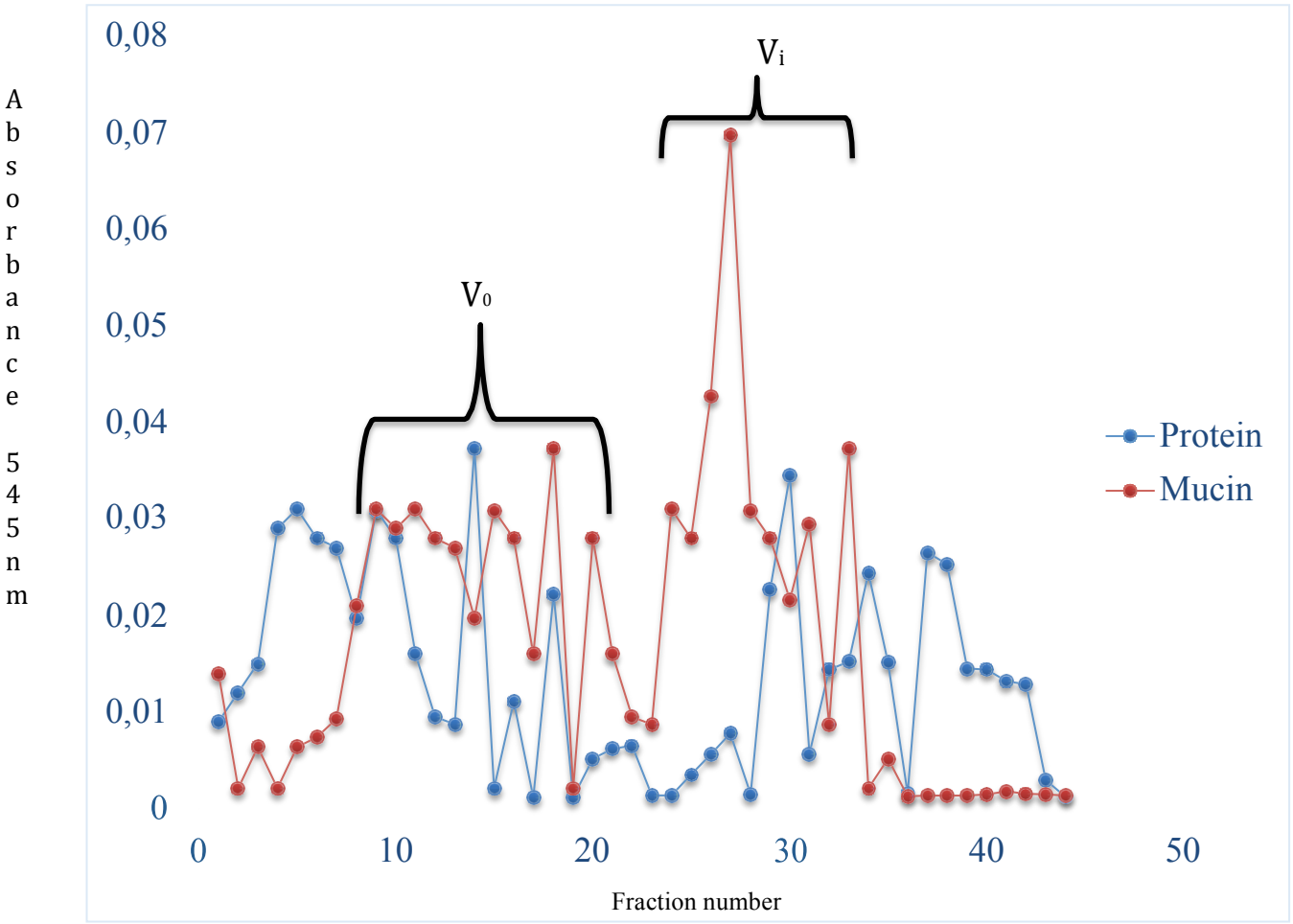


Figure 9 Sepharose CL-4B gel filtration profile of purified salivary mucin that was treated with DTT and alkylated with IAA. Samples were eluted with guanidium chloride buffer through a 40ml bed volume column and l ml fractions were collected, subjected to PAS and Bradford assay to measure the glycoprotein and protein content respectively. Due to the viscous nature of saliva the samples were extracted in 1:3 dilution with guanidium chloride buffer .

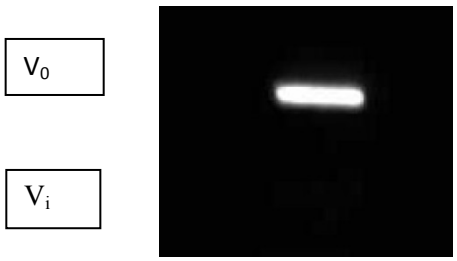
4.1.4 Confirmation of Mucin Identity

Slot Blot

The identity of the V_o and V_i peaks from the Sepharose CL-4B column bed was determined by the slot blot analysis. Briefly, the V_o and V_i fractions were vacuum blotted on a nitrocellulose membrane and probed with anti-MUC5B monoclonal primary antibody (Dallas Swallow, University College London) and anti-MUC7 polyclonal primary antibody (Santa Cruz Biotechnology), respectively. The samples tested showed immunoreactivity towards the primary antibodies indicating the presence of human salivary mucins in the test samples. Furthermore, our results revealed the identity of the V_o as MUC5B and the V_i fraction as MUC7. These results (Figure 10 a & b) showed that mucins were successfully separated and no contamination was observed.

Previously it has been shown that salivary mucins and the salivary glycoprotein SAG usually coprecipitates and elutes together as a complex hence the purification of salivary mucins has been of concern (Thornton et al. 2001). We investigated if no co-purification had occurred between our salivary mucins and SAG by probing with anti-gp340 monoclonal antibody (BioPorto Diagnostics) in all of the MUC5B and MUC7 samples. Our results (data not shown), showed no immunoreactivity in all the samples tested indicating that no co-purification had occurred.

a) Monoclonal Ab MUC5B



b) Polyclonal Ab MUC7

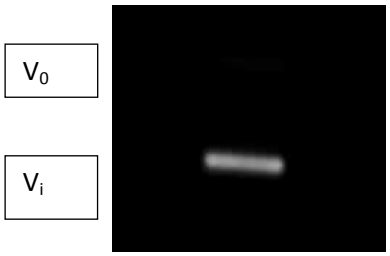


Figure 10 Slot blots of the PAS positive fractions (V_o and V_i) from the Sepharose CL-4B gel filtration column. The V_o (4a) and (4b) V_i samples represent MUC5B and MUC7 respectively.

4.1.5 Confirmation of Mucin Purity and Size Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

In order to visualize the mucin content in our crude and purified samples, the V_o and V_i fractions were subjected to SDS-PAGE analysis and stained with PAS. A characteristic feature of mucins is that it usually retains at the top the stacking gel due to their extensive carbohydrate side chains that accounts for 85% of mucins total molecular weight (Wu, Csako, and Herp 1994). This was observed in our crude sample (Figure 11). In addition, there was a smearing effect that was distinctly observed in crude saliva (Figure 11). PAS staining of crude saliva (Figure 11) showed two populations of different molecular weight sizes at approximately 250kDa and 180kDa, which were later, identified as MUC5B and MUC7 respectively.

MUC5B mobility was restricted at the top of the gel (Figure 12). We attributed this finding to its large molecular weight size and its extensive glycosylation. MUC7 (Figure 13) had faint bands of PAS staining as compared to MUC5B suggesting the possibility that the exposed regions of the mucin core were not highly glycosylated. Its molecular weight size was detected at 180kDa further supporting the theory that MUC7 is smaller compared to MUC5B (Thomsson 2002). In addition , more mobility was shown by MUC7 as more material entered the running gel (Figure 13).

No other bands were detected during the purification process confirming that mucin purification was successful and no contaminants were present. We observed slight variation in the mobility of the MUC7 among different samples. It is important to note

that due to the unavailability of aqua stain from our manufacturers, we did not conduct any aqua staining of our gels to visualize the protein content.

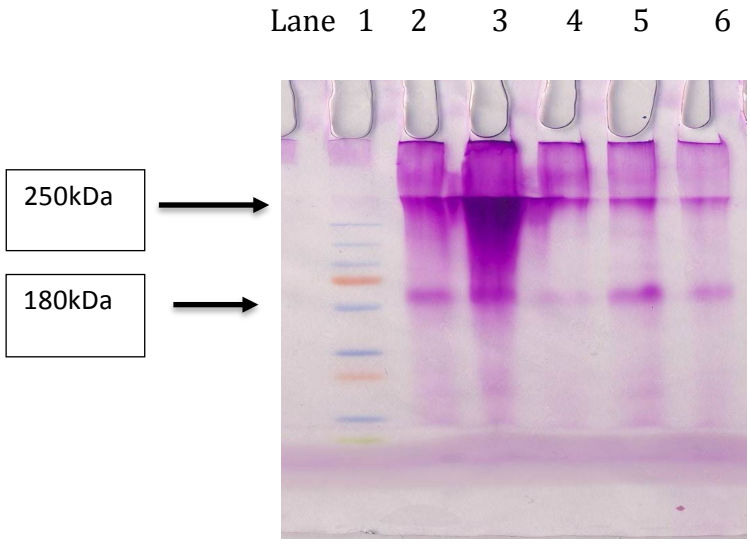


Figure 11 The presence of crude saliva proteins was detected using a 4-20% gradient SDS-PAGE gel followed by PAS staining of glycoproteins. The size of the salivary mucins was estimated using a molecular weight marker. Briefly, 1mg of lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 1 represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while Lane 2-6 shows crude saliva material.

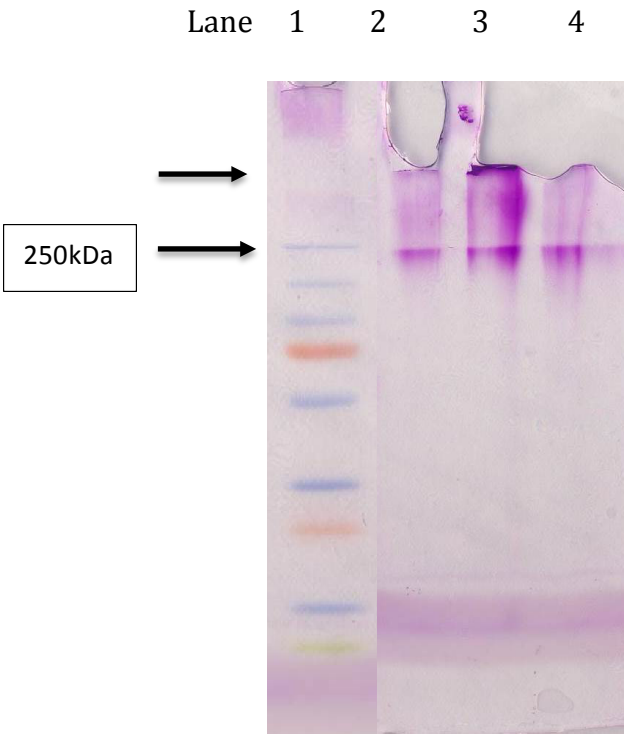


Figure 12 Estimation of mucin size and confirmation of the purity of MUC5B samples by 4-20 % gradient gel SDS-PAGE followed by PAS staining of mucin glycoprotein. Briefly, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 1 represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while Lane 2-4 shows 10 µl of purified V_0 material. The first arrow represents the start of the running gel and Lane 3 shows a dark band that could not penetrate the running gel and remained at the top of the well.

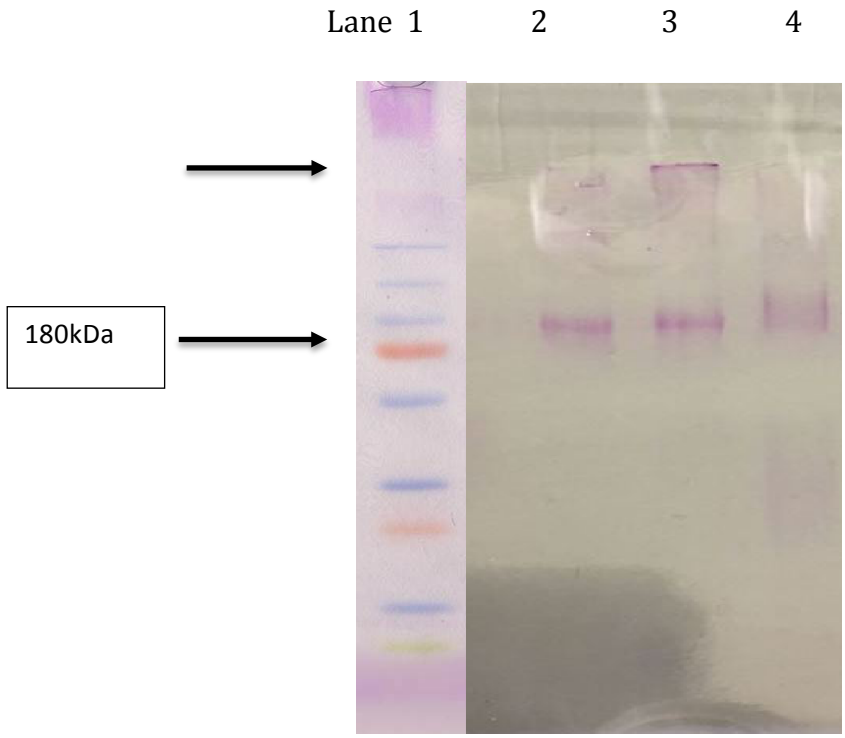


Figure 13 Estimation of mucin size and confirmation of the purity of MUC7 samples by 4-10 % gradient gel SDS-PAGE followed by PAS staining of mucin glycoprotein. Briefly, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 1 represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while Lane 2-4 shows 10 µl of the purified V_i material. The first arrow represents the start of the running gel .

4.1.6 Confirmation Of Treated Mucins Using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE).

Crude saliva and purified salivary mucins that were treated with either DTT or digested with trypsin were analysed by SDS-PAGE. Crude saliva that was treated with DTT showed degraded subunits of molecular size 130kDa and 55kDa while treatment of trypsin resulted in one distinct band of molecular size 250kDa (Figure 14 a &b) We concluded that treatment of crude saliva with DTT was effective as it led to an increase in mucin mobility indicative of degraded mucin subunits.

Purified samples that were treated with DTT (Figure 15a) or trypsin (Figure 15b) showed distinct bands of degraded subunits. DTT treated samples showed subunits that had molecular weights of 130kDa and 72kDa. These bands were distinct and the subunits showed polydispersity a characteristic of mucins. Treatment with trypsin showed distinct subunit bands of 250kDa and 130kDa. Most of the treated samples were restricted at the top of the gel and did not penetrate the running gel (Figure 15a & Figure 15b). In addition , very faint subunits of 72kDa in size were observed from DTT treated samples (Figure 15a)

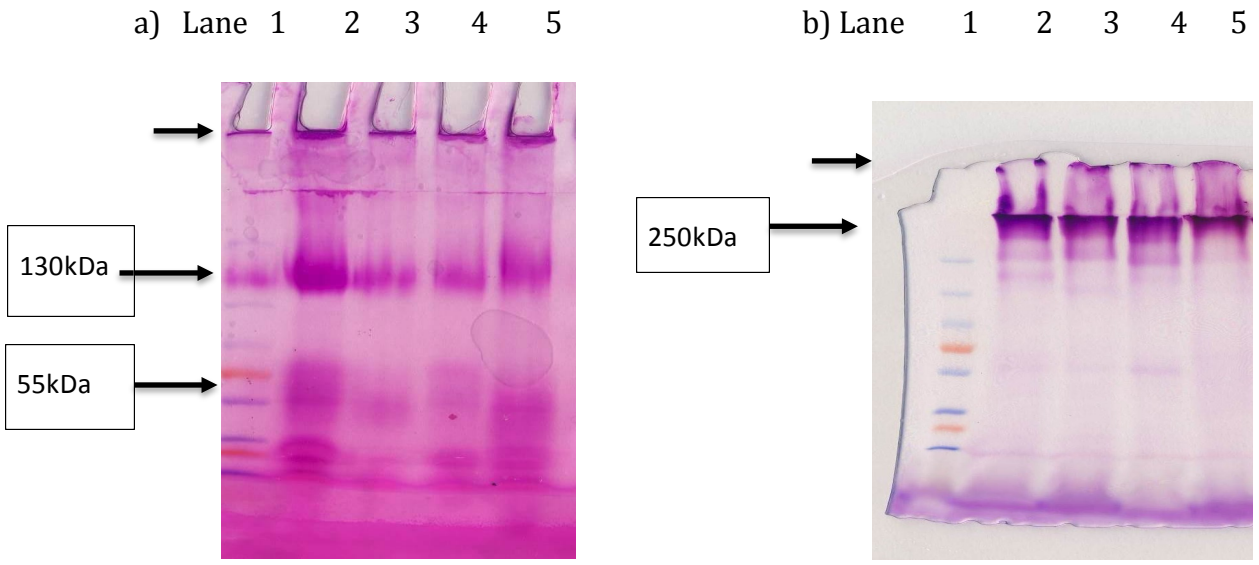


Figure 14 Estimation of mucin size and purity of crude that were treated with a) DTT and b) Trypsin on a 4-10 % gradient gel SDS-PAGE followed by PAS staining of mucin glycoprotein. Briefly, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 1 represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while Lane 2-6 shows 10 µl mucins that were treated with either a) DTT or b) Trypsin. The first arrow represents the start of the running gel on both pictures.

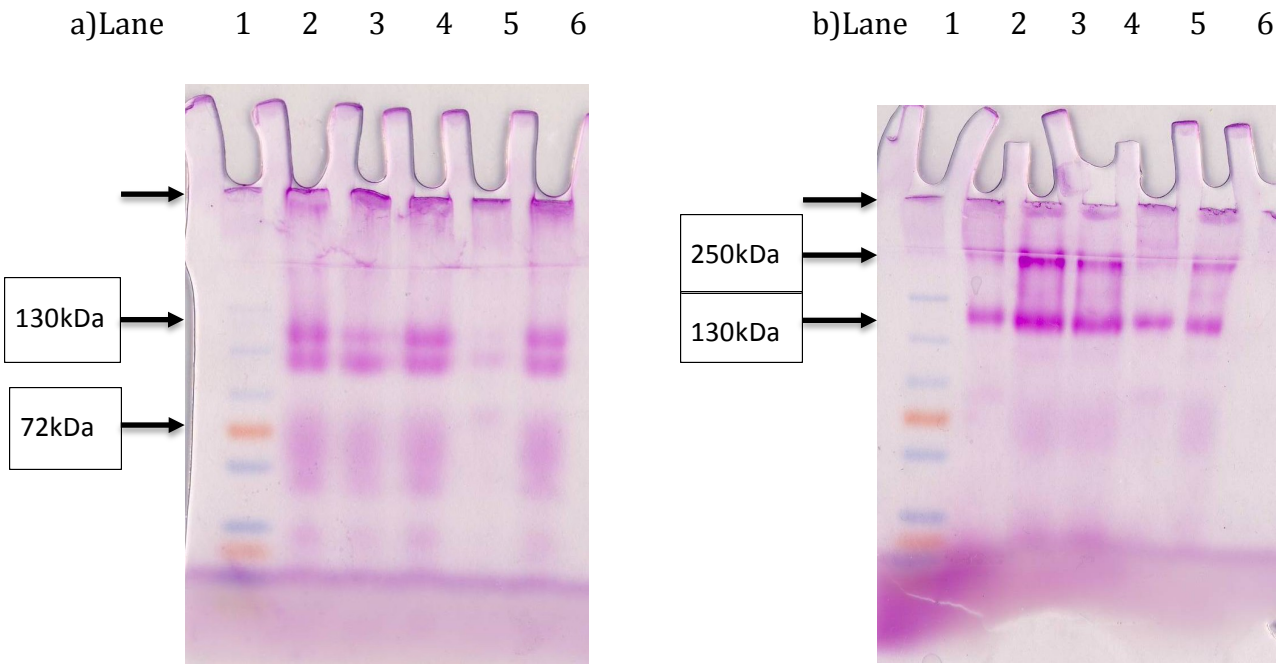


Figure 15 Estimation of mucin size and purity of purified salivary mucins that were treated with a) DTT and b) Trypsin on a 4-10 % gradient gel SDS-PAGE followed by PAS staining of mucin glycoprotein. Briefly, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 1 represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while Lane 2-6 shows 10 µl mucins that were treated with either a) DTT or b) Trypsin. The first arrow represents the start of the running gel on both pictures.

4.1.7 HIV-1 Neutralisation Assay

The anti-HIV-1 activity of crude saliva, MUC5B, MUC7, as well as trypsin digested and DTT reduced salivary samples was tested against the YU2 and DU422.1 pseudo virus strains. The inhibitory potential of these samples were calculated by measuring the IC_{50} defined as the concentration at which 50% of the virus is inhibited. The samples were considered non-inhibitory at the tested concentrations in cases where the neutralisation value was less than 30%, or the values were negative (regarded as artefacts) or where an increase in mucin concentration did not increase the inhibitory activity. These samples were assigned an arbitrary IC_{50} value of 2000ug/ml (triple the highest concentration used during these experiments) which was log transformed as it was impossible to extrapolate an IC_{50}. This value did not interfere with our statistical analysis as the data was non-parametric. Statistical analysis was done using the Kruskal Wallis test and the Mann-Whitney U test on all our samples.

Crude saliva, MUC5B and MUC7

From the cytotoxicity results (Table 1), crude saliva samples had IC_{50} that were as high as 1987 μg/ml to as low as 500 μg/ml. Most of the crude samples ranged between 500 μg/ml- 687 μg/ml. MUC5B had lower IC_{50} throughout all the samples with IC_{50} as low as 68 μg/ml and high as 710 μg/ml. MUC7 had three samples with IC_{50} that were >2000 and the remaining samples ranged between 65 μg/ml to 982 μg/ml.

There was a high inter-individual variability shown from our samples in their ability to neutralize the DU422 virus (Figure 16). A significant difference was detected by the non-parametric t test between crude saliva, MUC5B and MUC7 (Kruskal-Wallis, p=0.00025). Furthermore, pair wise analysis showed that MUC5B was more potent in inhibiting the DU422 virus as compared to crude saliva and MUC7 (Mann-Whitney U, p=0.0227 and p=0.0195 respectively). Furthermore, no difference was observed in inhibiting the DU422 virus by MUC7 and crude saliva (Mann-Whitney U, p=0.128).

Interestingly, the three cohorts of samples showed a significant difference in their anti-HIV activity against YU2 virus (Kruskal-Wallis, p=0.0078) (Figure 17). MUC7 showed a

higher inhibition compared to MUC5B and crude saliva (Mann-Whitney U, p=0.0341 and p=0.176 respectively). A significant difference in the inhibition of the YU2 virus was detected between MUC7 and crude saliva (Mann-Whitney U, p=0.0031).

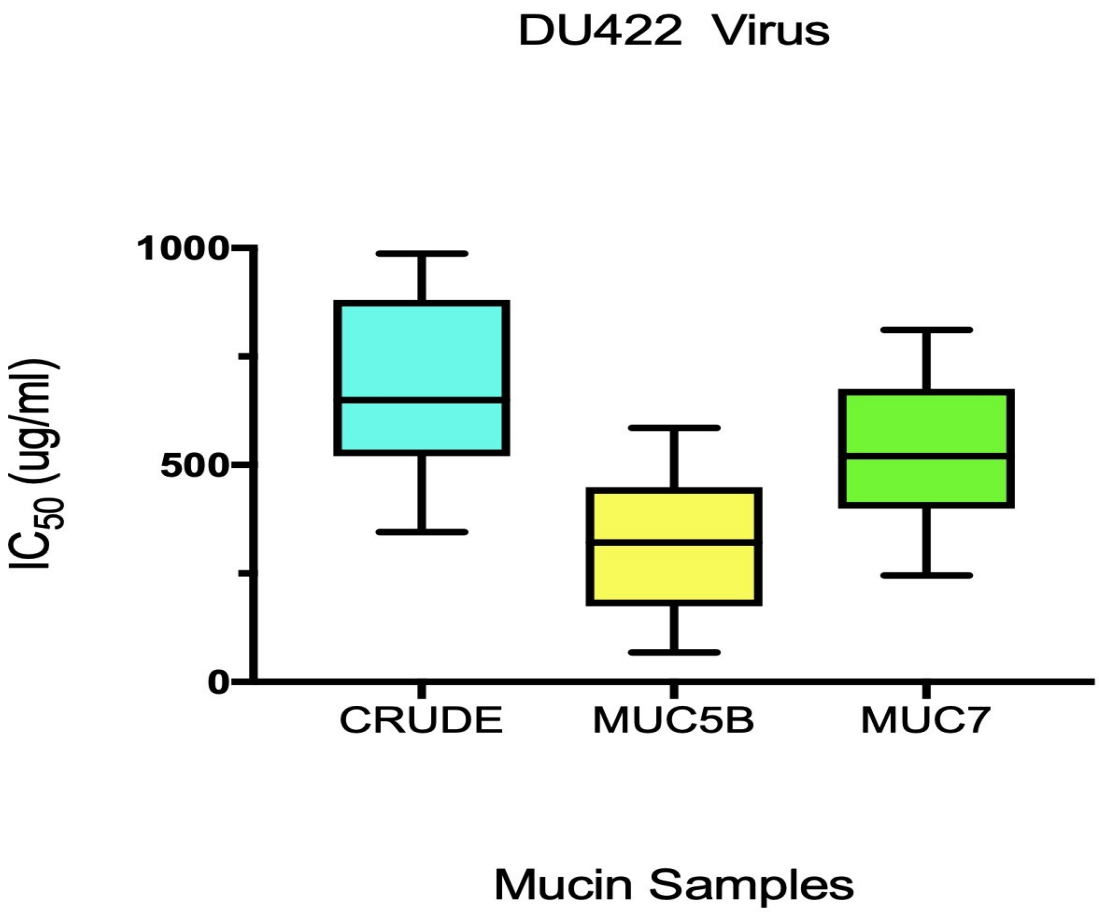


Figure 16 Comparison of the anti-HIV activity of crude saliva, MUC5B and MUC7 against the DU422 virus (Kruskal-Wallis, p=0.00025). MUC5B was more potent in inhibiting the DU422 virus as compared to crude saliva and MUC7 (Mann-Whitney U, p=0.0227 and p=0.0195 respectively). Furthermore, no difference was observed in inhibiting the DU422 virus by MUC7 and crude saliva (Mann-Whitney U, p=0.128).

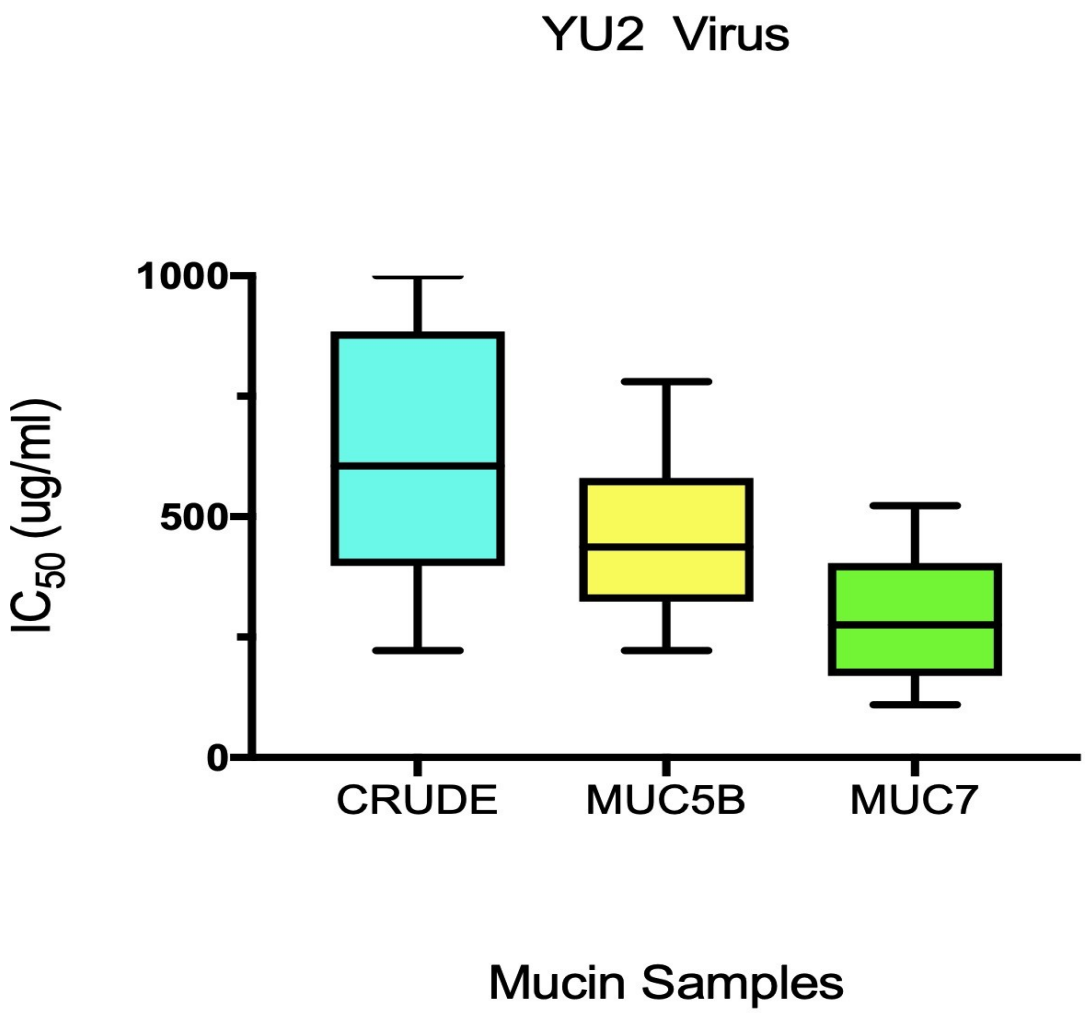


Figure 17 Comparison of the anti-HIV activity of crude saliva, MUC5B and MUC7 against the YU2 virus (Kruskal-Wallis, p=0.0078). MUC7 showed a higher inhibition compared to MUC5B and crude saliva (Mann-Whitney U, p=0.0341 and p=0.176 respectively). A significant difference in the inhibition of the YU2 virus was detected between MUC7 and crude saliva (Mann-Whitney U, p=0.0031)

DTT Treated saliva and DTT treated purified mucin

A significant difference was observed in the anti-HIV activity of the DU422 virus by DTT treated crude saliva and purified DTT treated salivary mucins(Figure 18) (Mann-Whitney U, p=0.006). Purified DTT treated samples showed a higher inhibitory activity compared to the DTT crude samples. The IC_{50} for both samples showed high variability against the DU422 virus. They ranged between 89 μg/ml to 956 μg/ml (Table 3)

However, despite the differences in IC_{50} between the DTT treated crude saliva and DTT purified mucin which ranged as low as 267 μg/ml to IC_{50} of 1000 μg/ml between the two cohorts, no significant difference in the inhibition of the YU2 virus by either sample was observed (Mann-Whitney U, p=0.06)(Figure 19).

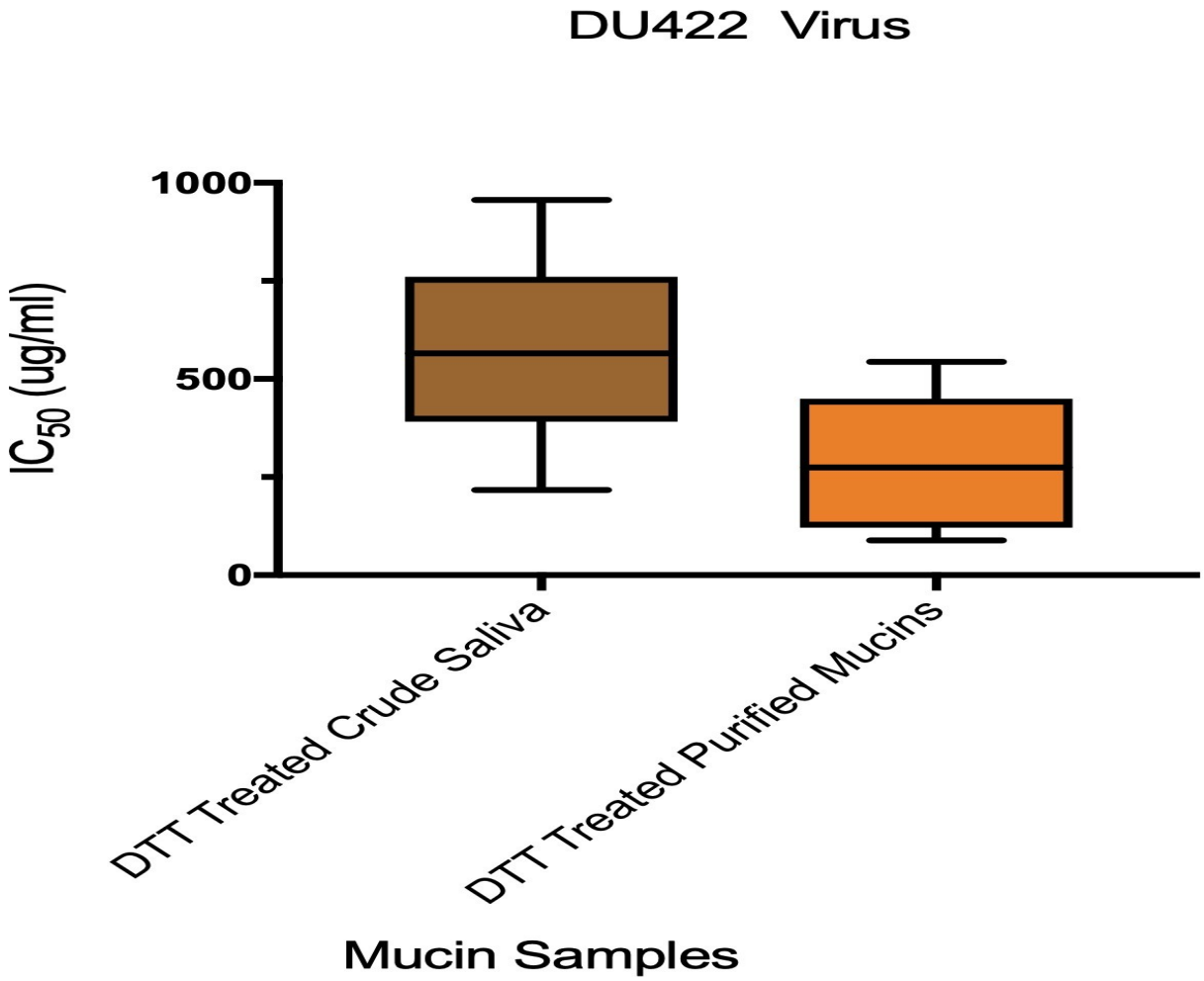


Figure 18 Comparison of the anti-HIV activity of DTT treated crude saliva and DTT treated purified mucins against the DU422 pseudo virus (Mann-Whitney U, p=0.006). Purified DTT treated samples showed a higher inhibitory activity compared to the DTT crude samples

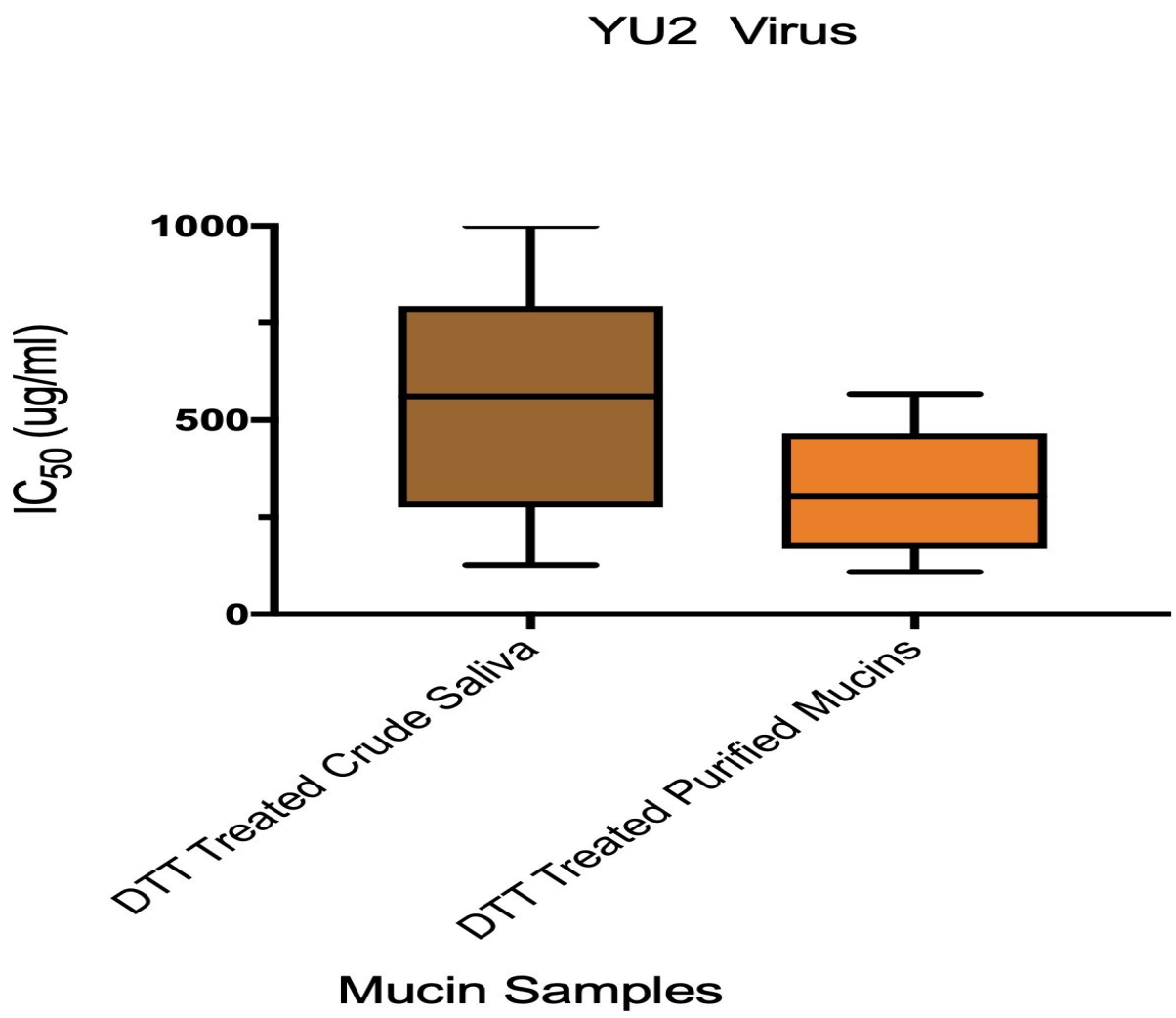


Figure 19 Comparison of the anti-HIV activity of DTT treated crude saliva and DTT treated purified mucins against the YU2 pseudo virus using TZMB/J cells (Mann-Whitney U, p=0.06). No significant difference was observed in the inhibition of YU2 pseudo virus by DTT treated crude saliva and DTT treated mucins.

Trypsin digested Saliva and trypsin digested purified mucins

Although purified mucins had better IC50s as compared to crude saliva, there was no significant difference observed in the anti-HIV activity of the DU422 virus by trypsin digested crude saliva and purified trypsin digested salivary mucins (Mann-Whitney U, p=0.08)(Figure 20). The IC_{50} for trypsin digested crude saliva samples showed high variability against the DU422 virus. They ranged between 900 μg/ml to 1610 μg/ml (Table 5). Purified digested samples had IC_{50} with less variation ranging from 800 μg/ml to 1400 μg/ml.

Despite the differences in IC_{50} between trypsin treated samples of crude saliva and purified mucin, no significant difference in the inhibition of the YU2 virus by either sample was observed (Mann-Whitney U, p=0.06) (Figure 21). The IC_{50} of purified mucins showed large variation ranging from 810 μg/ml to1653 μg/ml. Crude saliva that was digested with trypsin had IC_{50} ranging from 889 μg/ml to 1632 μg/ml(Table4.1 in appendix).

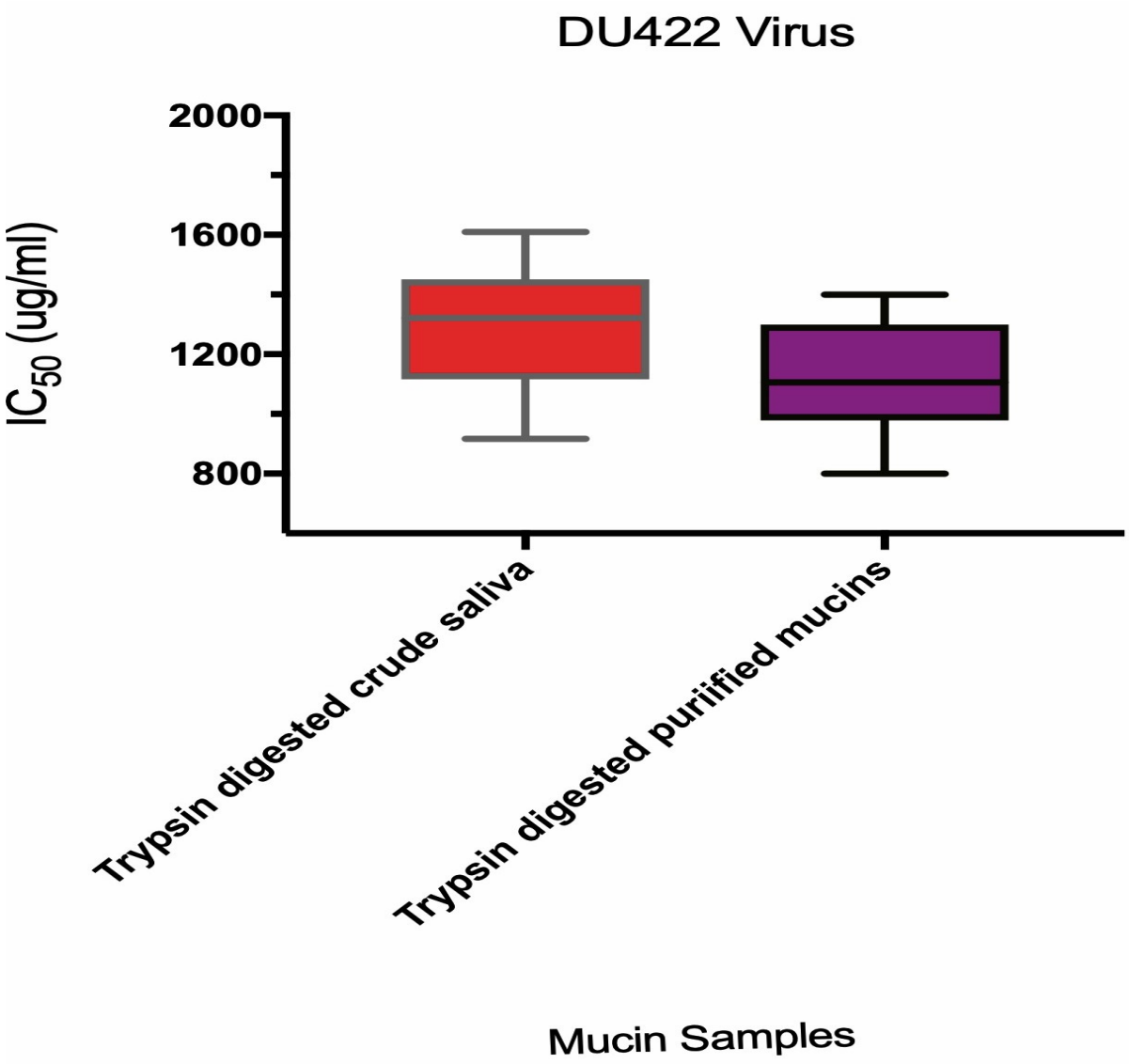


Figure 20 Comparison of the anti-HIV activity of trypsin digested samples between crude saliva and purified mucins . No significant difference observed in the anti-HIV activity of the DU422 virus by trypsin digested crude saliva and purified trypsin digested salivary mucins (Mann-Whitney U, p=0.08).

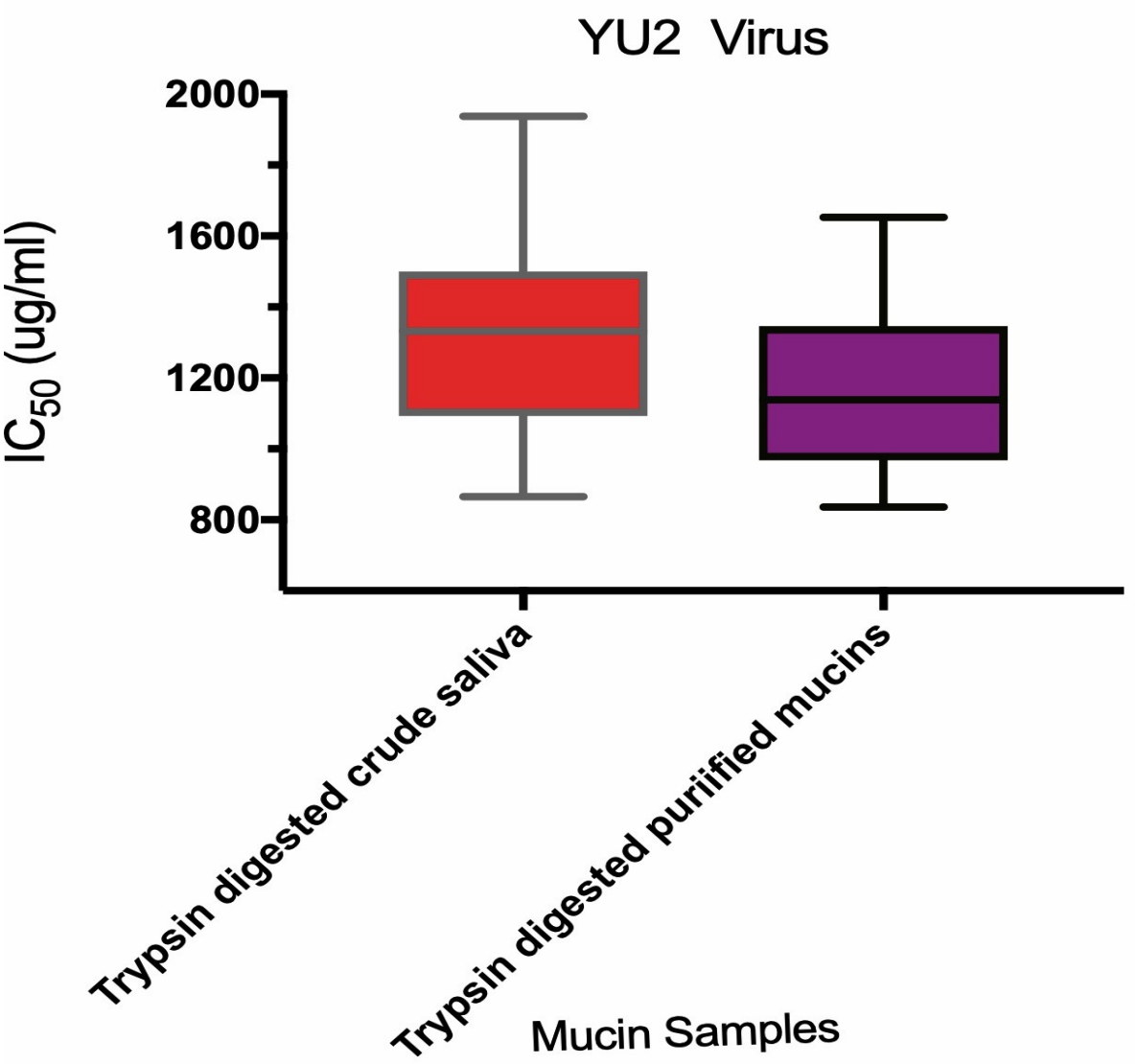


Figure 21 Comparison of the anti-HIV activity of trypsin digested samples between crude saliva and purified mucins . No significant difference observed in the anti-HIV activity of the YU2 virus by trypsin digested crude saliva and purified trypsin digested salivary mucins (Mann-Whitney U, p=0.06).

4.1.8 Cell Viability Assay

Crude and purified salivary samples that were used for the neutralization assay were tested or cytotoxicity using the MTT assay. If the cell viability remained above 70% and there was no increase in cell death with increase in sample concentration, mucins were considered non-toxic. In cases where the above condition was not met, the LC_{50} (50% Lethal dose) was calculated using the variable slope curve fit function in GraphPad Prism (La Jolla, CA, USA) which calculates the log(inhibitor) vs. normalized response (Table 1 & 2)

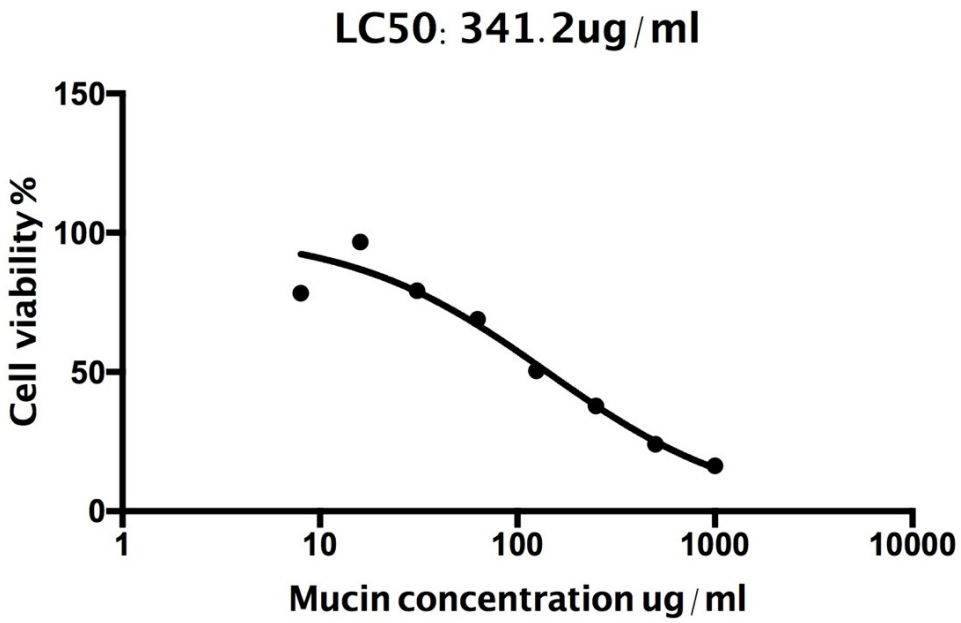


Figure 22 This graph demonstrates the criteria that was used to determine the cytotoxicity of TZM-bl/JC cells where an increase in mucin concentration resulted in an increase in cell death.

Crude, MUC5B and MUC7

In all the fifteen samples of crude saliva, eleven samples showed no detectable toxicity with an increase in crude saliva concentration (Table 1 & 2). The remaining four samples had LC_{50} ranging from 535µg/ml and as high as 1490µg/ml. Most samples of MUC5B and MUC7 showed no detectable cytotoxicity at the different concentrations that were being tested. The remaining samples of both MUC5B and MUC7 showed a high variation in LC_{50} that were high as >1287µg/ml and LC_{50} that were low as 167µg/ml. MUC7 in particular, showed more cell death as compared to MUC5B (Mann-Whitney U ,p-value=0.03)(Table 1 & 2).

Reduced Crude saliva and Reduced Purified Mucins

Initially, cell toxicity was detected across all the sample cohort of crude reduced saliva and purified reduced mucins. We hypothesised that the cytotoxicity of these samples was caused by DTT and IAA that was not completely removed during dialysis and that it could be alleviated if we dialysed our samples again against PBS. To test this, we cultured TZM-bl/J cells and tested our dialysed samples at different concentrations. Four out of nine of our samples showed detectable toxicity while the remaining samples showed a large variation ranging from LC_{50} high as 39 876 µg/ml and LC_{50} low as 385µg/ml (Table 5 in appendix). We therefore dialysed all our samples with PBS to exclude interference of cytotoxicity in our analysis.

Trypsin digested saliva and Trypsin digested Purified Mucins

The majority of the trypsin digested saliva and trypsin digested purified salivary mucin samples showed no detectable toxicity while the remaining samples showed a huge variation with the LC_{50} values ranging from 1870µg/ml to 20432µg/ml (Table 5.1 in appendix)

Table 1 Cytotoxicity (LC50) and HIV-1 neutralization values of DU422 virus (IC50) by crude saliva and purified MUC5B and MUC7 mucin samples

Samples	CRUDE		MUC5B		MUC7	
	LC50 (µg/ml)	IC_{50} µg/ml	LC50 (µg/ml)	IC_{50} µg/ml	LC50 (µg/ml)	IC_{50} µg/ml
1	No toxicity	687	No toxicity	128	538	455
2	No toxicity	643	No toxicity	321	769	354
3	No toxicity	687	No toxicity	189	No toxicity	>2000
4	No toxicity	566	No toxicity	68	No toxicity	>2000
5	No toxicity	500	1209	322	No toxicity	>2000
6	No toxicity	345	567	132	No toxicity	982
7	No toxicity	875	No toxicity	421	1287	438
8	758	987	No toxicity	531	605	321
9	No toxicity	656	No toxicity	332	465	476
10	No toxicity	527	No toxicity	585	167	656
11	No toxicity	1345	No toxicity	315	534	564
12	No toxicity	1987	No toxicity	710	715	641
13	535	897	No toxicity	432	No toxicity	734
14	625	1308	No toxicity	68	No toxicity	245
15	1490	888	No toxicity	178	239	811

Table 2 Cytotoxicity (LC50) and HIV-1 neutralization values of YU2 virus (IC50) by Crude saliva and purified MUC5B and MUC7 mucin samples

Samples	CRUDE		MUC5B		MUC7	
	LC50 (µg/ml)	IC50 µg/ml	LC50 (µg/ml)	IC50 µg/ml	LC50 (µg/ml)	IC50 µg/ml
1	No toxicity	629	No toxicity	185	538	267
2	No toxicity	No inhibition	No toxicity	333	769	138
3	No toxicity	678	No toxicity	411	No toxicity	198
4	No toxicity	782	No toxicity	439	No toxicity	65
5	No toxicity	651	1209	178	No toxicity	57
6	No toxicity	433	567	410	No toxicity	324
7	No toxicity	529	No toxicity	611	1287	115
8	758	342	No toxicity	484	605	778
9	No toxicity	744	No toxicity	382	465	607
10	No toxicity	880	No toxicity	102	167	213
11	No toxicity	761	No toxicity	1091	534	879
12	No toxicity	907	No toxicity	381	715	221
13	535	767	No toxicity	32	No toxicity	111
14	625	1809	No toxicity	648	No toxicity	84
15	1490	668	No toxicity	478	239	216

Table 3 Cytotoxicity (LC50) and HIV-1 neutralization values of DU422 virus (IC50) by DTT treated crude saliva and DTT treated purified mucins.

	DTT Treated crude saliva		DTT Treated Purified Mucins	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1000	No toxicity	448
2	No toxicity	278	No toxicity	567
3	No toxicity	622	No toxicity	109
4	No toxicity	776	No toxicity	250
5	No toxicity	333	1209	411
6	No toxicity	500	567	109
7	No toxicity	846	No toxicity	522
8	758	267	No toxicity	355
9	No toxicity	667	No toxicity	189

Table 4 Cytotoxicity (LC50) and HIV-1 neutralization values of YU2 virus (IC50) by DTT treated crude saliva and DTT treated purified mucins.

	DTT Treated crude saliva		DTT Treated Purified Mucins	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	956	No toxicity	500
2	No toxicity	564	No toxicity	544
3	No toxicity	841	No toxicity	299
4	No toxicity	734	No toxicity	225
5	No toxicity	567	1209	125
6	No toxicity	650	567	335
7	No toxicity	411	No toxicity	433
8	758	333	No toxicity	689
9	No toxicity	217	No toxicity	250

Table 5 Cytotoxicity (LC50) and HIV-1 neutralization values of YU2 virus (IC50) by trypsin digested crude saliva and trypsin digested purified mucins.

	Trypsin digested crude saliva		Trypsin digested purified mucins	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1190	No toxicity	900
2	No toxicity	1087	No toxicity	1632
3	No toxicity	1233	No toxicity	917
4	No toxicity	1467	No toxicity	1021
5	No toxicity	1306	1209	1161
6	No toxicity	889	567	1332
7	No toxicity	1312	No toxicity	1421
8	758	1653	No toxicity	810
9	No toxicity	1085	No toxicity	1332

Table 6 Cytotoxicity (LC50) and HIV-1 neutralization values of DU422 virus (IC50) by trypsin digested crude saliva and trypsin digested purified mucins.

	Trypsin digested crude saliva		Trypsin digested Purified mucins	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1610	No toxicity	1221
2	No toxicity	1161	No toxicity	999
3	No toxicity	1100	No toxicity	917
4	No toxicity	1421	No toxicity	1100
5	No toxicity	1321	1209	1400
6	No toxicity	1121	567	800
7	No toxicity	1543	No toxicity	1099
8	758	1332	No toxicity	1332
9	No toxicity	917	No toxicity	1111

4.2 DISCUSSION

Previous studies in our laboratory has shown that crude saliva, purified salivary mucins together with purified mucins from breast milk and the cervix inhibits HIV-1, whereas crude breastmilk and cervical mucus showed no inhibition (Habte et al. 2006:Habte et al. 2008; Peacocke 2011; Peacocke et al. 2012; Mthembu et al. 2014).This study aimed to determine the minimum peptide chain length and structure of a gel forming mucin that retains the anti-HIV-1 activity. Furthermore, this study aimed to investigate the role of glycans in the inhibition of HIV-1. Salivary samples used in this study were obtained from HIV-negative individuals who declared a risk free lifestyle.

Saliva was collected in 6M GuHCL with PI and was successfully purified using caesium chloride gradient ultracentrifugation a well-established method for purifying mucins (Creeth and Denborough 1970; Mall 1987; Mall 1988). These samples underwent two caesium chloride gradient ultracentrifugation spins in-order to remove the excess proteins and contaminates before yielding mucins (Figure 4). Thereafter, the salivary glycoproteins were successfully separated on a Sepharose CL-4B bead (Figure 5). The V_o fraction which was later identified as MUC5B eluted in the first peak whilst the V_i fraction identified as MUC7 eluted second and had a smaller peak compared to the V_0 peak (Figure 5).

Crude and purified samples that were treated with DTT or digested with trypsin were subjected to gel filtration to monitor the changes in mucin size. In both treatments of crude and purified salivary samples, there was incomplete digestion and reduction of the samples with trypsin and DTT respectively (Figure 6,7, 8 & 9). A rightward shift in mucin peak is an indicator for reduction in mucin size (Allen 1989). Our gel filtration profiles did not shift to the right and the size of peaks did not decrease as expected (Figure 6, 7, 8 & 9). We attributed this to GuHCl, a denaturant that was used as the extraction buffer. The presence of protease inhibitors in the buffer could have prevented any endogenous proteolysis to take place. In addition, the aggregatory properties of GuHCl could have

masked the protein naked regions or the disulphide bridges within the mucins thus making it difficult for digestion and reduction to occur (Mall 1984). Moreover, the digestion of saliva using trypsin was not pH controlled and this could have affected the optimal rate at which proteolytic digestion occurred.

Antibodies raised against the human mucin MUC5B and MUC7 were used during slot blot analysis. The purification of salivary mucins has raised some concern since it was previously identified by Thornton et al. (2001) that salivary mucins and SAG precipitates together and elutes during purification as a complex. To solve this, we tested all our samples with a monoclonal antibody anti-gp340 and no immunoreactivity was detected. These results (Figure 10) showed that our mucins were successfully separated when the V_0 and V_i fractions were tested against their respective antibodies. SDS Page analysis followed by PAS staining of crude saliva showed two population of mucins of molecular weights size 250kDa and 180kDa (Figure 11). The smear effect observed in all the lanes in figure 11 attributed to the heterogeneity and polydispersity of glycoproteins (Harding, 1984). Briefly, heterogeneity is a feature that results when molecules do not have a distinct single molecular weight while polydispersity occurs when a molecule has different densities which results in different molecular weights (Harding, 1984).

Regardless of mucin samples showing the same purification profiles, there were differences in electrophoretic mobility of MUC5B and MUC7 (Figure 12 & 13). In addition, the mucin yield that was obtained after the purification process differed from individual to individual. The difficulty in getting volunteers to consistently spit saliva at specific time intervals and diurnal variations in salivary glands could contribute to the differences in mucin yield and mobility.

Subtype C is the most prevalent strain in South Africa and has a high global predominance whereas the subtype B virus is not as common in Africa. The subtype B strain is most common and prevalent in Europe and Asia (Junqueira et al. 2016). The YU2 virus and the DU422 virus which are both laboratory adopted subtype B and C strains respectively, were used for the neutralization assays in this study to determine the role of salivary

mucins in the inhibition of HIV-1. To the best of my knowledge, this was the first time that our laboratory conducted neutralization assays on a subtype B strain. The results of this present study and that of Peacocke et al. (2012) agree that both crude and purified salivary mucins from HIV-negative individuals inhibit HIV-1 *in vitro.* While successfully demonstrating this, the study by Peacocke et al. (2012) did not quantitatively compare the inhibitory potential of the mucin samples. Therefore, this present study used a more advanced neutralisation assay.to calculate dose response curves which measured the potency (IC_{50s}) of the mucin samples.

The traditional neutralization method used by Habte et al. (2006; 2010) and Peacocke et al. (2012) involved the use of mitogen-stimulated peripheral blood mononuclear cells (PBMCs) and a p24 antigen to detect the replication of the HIV-1 virus. While this assay was considered to be of physiological value, there was a major concern regarding reproducibility (Polonis et al. 2008). The producibility of the p24 assay was affected by using PBMCS from different donors. This is the reason for the considerable variability detected with using this assay during experiments (Montefiori and Evans 1999). The differences in the number of CD4+ cells and CD4+ surface cell receptors on PBMC cells , from different individuals could affect their susceptibility to HIV-1. Moreover, genetic polymorphism in chemokine receptors as well as host cell surface receptors could also account for the variation that this assay has (Polonis et al. 2008).

In this present study we used the luciferase reporter assay as described by Montefiori (2009). This assay involved the infection of a pseudoviral strain to a transformed cell line such as the TZM-bl/JC cells. This cell line express the CD4, CXCR4, ICAM 3 and MHC class ll receptors making them suitable host cells for HIV infection (Nara et al. 1987). In addition, these cells are modified and contain reporter genes for luciferase and E. coli β-galactosidase which are initiated in the presence of the HIV Tat. Viral infection is indicated by the activity of the luciferase (Montefiori 2009). The envelope protein was cloned and incoperated into a plasmid that contained a dysfunctional env gene sequence. The advantage of using this dysfunctional env gene is that it allows for the host cells to be infected only once thereby making it a good alternative to use in the laboratory

than a wildtype virus (Montefiori 2009). Moreover, the env plasmids were engineered with viruses from different clades making it easier to test the anti-HIV-1 activity of different strains. This assay is safe, needs less time to be conducted and is reproducible. Moreover, inter-experimental comparisons are easier because of the platform used to give standardized results (Montefiori 2005; Montefiori 2009; Sarzotti-Kelsoe et al. 2014). Although the TZM-bl/JC cell line has a high expression of the CCR5 co-surface receptors , this may not be a true representation of the cellular environment *in-vivo*. Nonetheless, this assay closely mimics the natural physiological conditions.

The results of this present study showed that crude saliva, MUC5B and MUC7 has the ability to inhibit the transmission of HIV-1 to TZM-bl/JC cells by HIV-1 DU422.1 and YU2 pseudo-virus strain. While MUC5B showed a higher inhibitory potential against the DU422 virus, MUC7 had a greater anti-HIV-1 activity against the YU2 pseudo-virus strain. This finding requires further investigation. Furthermore, crude saliva showed comparable HIV-1 inhibition against both the DU422 virus and the YU2 virus.

As shown in figure 16 & 17 crude saliva inhibited the infection of TZM-bl/JC cells against the DU422 and YU2 pseudo-virus strains (Kruskal-Wallis, $p=0.00025$). This is in agreement with the initial findings of Fultz (1986) and Fox et al (1990) in which saliva inhibits the activity of HIV-1 *in-vitro.* The isotonic nature of saliva together with the presence of immunological factors and non-immunological proteins in the oral cavity accounts for the inhibitory potential of saliva towards HIV-1 pseudo-virus strains (Matsuda et al. 1993; Artenstein et al. 1997; Yasida et al. 1998). This also explains why the transmission of HIV via the oral cavity is rare. In addition, the presence of soluble factors such as SPL1 and lactoferrin in saliva are known to play a crucial role in the inhibition of HIV-1. While SLPI interacts with host cells thereby preventing viral binding and subsequent infection (McNeely et al. 1995; McNeely et al. 1997; Ma et al. 2004), lactoferrin is known to inhibit one of the HIV-1 infection cycle enzymes, reverse transcriptase (Ng et al. 2001). These proteins in saliva provide Synergistic effects in their role as inhibitors against the transmission of HIV-1 (Yeh et al. 1992; Kazmi et al. 2006).

MUC5B showed the highest anti-HIV-1 potency against the DU422 virus between the three cohorts. These results agree with the initial findings of Habte et al. (2008; 2010) ; Peacocke et al. (2012) and McQuaid 2017 (manuscript under review) in which MUC5B showed a higher potency against the DU422 pseudo virus compared to MUC7. The finding that MUC5B shows greater potency compared to MUC7 is in line with the theory that carbohydrate side chains play a vital role in their anti-HIV-1 activity. The carbohydrates moieties on MUC5B are large, many and extensive as compared to MUC7 which could account for an effective aggregatory activity (Thornton et al. 1999; Levine et al. 1987). They account for 78% of the total molecular weight of MUC5B (Levine et al. 1987). The threonine and serine residues that form the tandem repeat sites of MUC5B contain many potential sites for O-linked glycosylation (Zalewska et al. 2000). The higher glycosylation seen on MUC5B could increase the glycan /viral binding sites or could increase the net negative charge of mucins which in-turn could increase its aggregatory function. The different glycosylation profiles shown by both mucins (MUC5B and MUC7) may account for the differences observed during viral inhibition (Thomsson et al. 2002).

In addition, MUC5B consists of many subunits that are joined together via disulphide bonds. These subunits add to the polymeric nature and the gel forming abilities of MUC5B. Furthermore, these subunits physically block the HIV-1 particles ensuring no infection of target cells occur (Zalewska et al. 2000; Sellers et al. 1988). MUC7 on the other hand, primarily consists of monomeric subunits with less sugar side chains (Mehrotra, Thornton and Sheehan 1998). This could account for the lower aggregatory potential towards the DU422 pseudo-viral strain. While this is based on the understanding that the inhibition of HIV-1 by saliva and salivary mucins is thought to be primarily by aggregation (Archibald and Cole 1990; Malamud et al.1993; Bergey at al. 1994), the limited inhibitory activity of MUC7 contradicts earlier studies which demonstrated MUC7 as the most dominant glycoprotein in the inhibition of HIV-1 via salivary secretions (Bergey et al. 1994; Nagashunmugan et al. 1998). Perhaps *In vivo,* MUC7 form complexes with other soluble proteins to elicit an efficient anti-HIV-1 response. It is possible that the purification of MUC7 diminishes this response. Future experiments should use electron microscopy work to determine the mucin/virus interaction as well as the mucin/cell and virus interactions of MUC5B and MUC7 to account for these inhibitory differences.

There has been a major debate as to whether the aggregatory activity of salivary mucins is specific or non-specific. The presence of infectious viral and bacterial agents in saliva such as hepatitis B virus, herpes simplex virus (HSV), HIV-2 (Tabak 1995; Bosch et al. 2000; Crombie et al. 1998) in saliva suggests the possibility that the aggregatory activity could be specific. In addition, Bobek and Situ (2003) reported that the binding of salivary mucins to pathogens prevents them from binding to targeted host cells and subsequently causing infection. Furthermore, saliva was demonstrated to selectively bind to and clear both viral and bacterial microorganisms from the oral cavity (Prakobphol et al. 2005).

The interaction of MUC5B and MUC7 towards viruses and bacteria differs which suggests the possibility that they have different mechanisms through which they interact and inhibit HIV-1 (Frenkel and Ribbeck 2015a). It is also known that MUC7 demonstrates better bacterial binding while MUC5B shows effective viral binding properties (Frenkel and Ribbeck 2015a). This current study showed that mucins interact with viral strains differently. MUC7 showed a better anti-HIV activity against the YU2 pseudo-virus as compared to DU422 virus (Mann-Whitney U, $p=0.0031$) Although the mechanism through which MUC7 inhibits the YU2 pseudo-virus is unknown, we speculate that the observation by Bobek and Situ (2003); Smith and Bobek (2001) could account for this. The authors found that the presence of cationic peptides together with the histatin 5 peptides that were present on the N-terminal residues of MUC7 offered an added advantage that specifically allowed MUC7 to entrap viruses causing an effective inhibitory activity. Furthermore, MUC7 has been associated with specific bacterial strains such as streptococci strain (Murray et al. 1982; Ligtenberg et al.1992; Murray et al. 1992). Tabak (1990) demonstrated that saliva that was depleted of MUC7 and not MUC5B could cause agglutination and subsequently abolish the activity of certain streptococci strains. Moreover, he demonstrated that sialic acids present on the MUC7 glycans were involved in the binding of different bacteria including Streptococcus gordonii and Streptococcus sanguis strains (Murray et al. 1992; Levine et al. 1978). Thus, the structural difference between MUC5B and MUC7 could account for the differences observed during inhibition. MUC7 could be involved in different mucin/ virus interactions with different strains and this could account for the differences observed inhibition.

In-order to assess the minimum size that is required for mucins to retain their anti-HIV-1 activity, crude saliva together with purified mucins were treated with either DTT or 0.25% trypsin and tested against the two different pseudo-virus strains. DTT treatment breaks down the disulphide bonds that links mucin subunits together via the cysteine residues (Carlstedt et al. 1983a). Purified salivary mucins that were treated with DTT had the highest anti-HIV-1 activity towards both strains compared to crude saliva that was treated with DTT (Mann-Whitney U, p=0.006) (Figure 18 & 19). This observation suggests that even when the gel forming properties of mucins are compromised, mucins still retain their ability to inhibit viruses (Carlstedt et al. 1983a). However, the reduced inhibitory potential in samples that were treated with DTT compared to samples that were not treated(both crude and purified saliva) suggests the possibility that disulphide bonds may play a role in recognizing viral epitopes (Pillay 2017 thesis).

Trypsin digestion of both crude saliva and purified salivary mucins yielded minimal inhibition of both DU422 and YU2 pseudo-viral strain compared to reduced subunits (Figure 20 & 21). While it is possible that extensive glycosylation of mucins could have masked the interaction of the trypsin enzymes to the underlying protein core , it is highly likely that this could have affected any interactions between the peptides and the viral particles thereby influencing the potency of these samples towards the different pseudo-strains (Wu, Csaka and Herp 1994). Furthermore, given that our extraction buffer, GuHCl is known to cause aggregation (Mall 1984), the peptide fragments could have aggregated masking the viral binding sites which subsequently lead to a reduced inhibitory activity. However, the inhibition shown by these peptide fragments suggests the possibility that mucins do not have to be large or polymeric, they just need to have the required number of oligosaccharides or the specific sugar side chain sequence and composition to inhibit HIV-1 via aggregation.

The average concentration of MUC5B and MUC7 in saliva under mastication conditions as reported by Rayment et al. (2000) is 233ug/ml and 133ug/ml respectively. During stimulation, parotid secretions increase considerably (Humphrey and Williamson 2001). However, since parotid secretions are devoid of mucins, the above concentrations are less likely to represent the total mucin concentration in whole saliva. Ten of the 30 Purified MUC5B (Table 1 & 2) had IC50 below the reported physiological concentrations. Only 5 purified samples of MUC7 (Table 1 & 2) had IC50 at or below the reported values. The

values reported by Rayment et al. (2000) had high standard deviations these values were used to give context rather than being used as absolute values.

The cytotoxicity of crude saliva and purified salivary mucins were tested against the TZM-bl/JC cells using the MTT assay. If the cell viability remained above 70% and there was no increase in cell death with increase in sample concentration, mucins were considered non-toxic. In cases where the above condition was not met, the LC_{50} (50% Lethal dose) was calculated using the variable slope curve fit function in GraphPad Prism (La Jolla, CA, USA) which calculates the log(inhibitor) vs. normalized response (Figure 6). Our samples showed that mucins were not toxic hence we ruled out the possibility that salivary mucin samples could cause cell death. Overall, most of the crude saliva samples together with purified MUC5B samples were non-toxic and gave IC_{50} values that validated the neutralisation assay (Table 1 & 2). The majority of MUC7 and DTT reduced samples showed cytotoxicity and while it is possible that residual traces of DTT and mucin extraction buffer GuHCl caused cell death, this is unlikely since all samples were subjected to extensive dialysis. Therefore, using a different cell line in future experiments might be valuable in this regard.

There is a significant decline in the amount and concentration of salivary mucins in older people as compared to younger individuals (Bolscher et al. 1999; Denny et al. 1991). Furthermore, the sugar content between the two-age group also differs. Tabak (1982) demonstrated that the amount of sugar content of salivary mucins present in adults is twice as much as compared to the younger group. In addition, the frequent use of medication and the loss of function of the submandibular parenchyma is argued to cause a decrease in the amount, concentration and potency of these mucins in the older aged group (Denny et al. 1991). Therefore, future studies should put to account the age difference when testing the anti-HIV activity of crude saliva and purified salivary mucins against the different viral strains.

The results of this study shows that salivary mucins inhibit the activity of HIV-1. In addition, MUC5B a gel forming mucin showed greater anti-HIV-1 activity towards the DU422 virus, a subtype C strain than MUC7. Furthermore, MUC7 had a significantly

higher inhibitory potency towards the YU2 virus, a subtype B strain. However, it should be noted that this present study did not test the anti-HIV activity of salivary mucins against a wide range of subtype B strains hence we cannot conclude if this is a characteristic of the YU2 virus or of all subtype B strains. Thus, future studies with large sample sizes need to investigate if MUC7 display such potency towards different strains of the subtype B virus.

CHAPTER FIVE: A COMPARISON OF THE ANTI-HIV-1 INHIBITORY ACTIVITY OF DEGLYCOSYLATED MUC5B AND MUC7

5.1 Introduction

The addition of sugar chains to a protein core (glycosylation) is a highly conserved type of protein modification and requires a lot of enzymes (Hagen et al. 2013). To date, two forms of mucin glycosylation, N-linked and O-linked occur on most secreted or membrane bound proteins. O- linked mucin glycosylation is an evolutionarily conserved protein modification that is found in most mammals, worms and in certain insects (Hagen et al 2013). This modification is characterised by the addition of N-acetylglucosamine (GALNAc) to the hydroxyl group of serine or threonine residues (Rose and Voynow 2005). N-linked oligosaccharides are usually attached via N-Acetylglucosamine linkage to asparagine (Jansen et al. 2009).

In addition to N-acetyl galactosamine (GalNAc), N-acetyl glucosamine (GlcNAc), N-acetyl neuraminic acid, fucose and galactose are the sugars that make up the carbohydrate moiety of O-linked mucins. This moiety accounts for 85% of their molecular weight (Wu, Csako, and Herp 1994) and serves to protect the mucin protein core (apomucin) from proteolysis and degradation (Wu, Csako, and Herp 1994). Moreover, the extensive glycosylation serves to extend the protein backbone transforming it into a globular structure (Shogren et al 1989). The sequence and extent of glycosylation depends on the type, function and localisation of the mucin (Sellers et al. 1988). In addition, the length of these glycan chains also varies. The terminal residues of mucins are made up of sialic acids, fucose and sulphated GlcNAc or blood group determinants that give mucins their hydrophobic and hydrophilic properties (Rose and Voynow 2005). Table 7 shows the structures of O-glycan cores and antigenic epitopes found in mucins.

Table 7 The O-glycan core structures and antigenic epitopes found in mucins (taken from Brockhausen et al. 2009).

O-Glycan	**Structure**
Core	
Tn antigen	GalNAcαSer/Thr
Sialyl-Tn antigen	Siaα2-6GalNAcαSer/Thr
Core 1 or T antigen	Galβ1-3GalNAcαSer/Thr
Core 2	GlcNAcβ1-6(Galβ1-3)GalNAcαSer/Thr
Core 3	GlcNAcβ1-3GalNAcαSer/Thr
Core 4	GlcNAcβ1-6(GlcNAcβ1-3)GalNAcαSer/Thr
Core 5	GalNAcα1-3GalNAcαSer/Thr
Core 6	GlcNAcβ1-6GalNAcαSer/Thr
Core 7	GalNAcα1-6GalNAcαSer/Thr
Core 8	Galα1-3GalNAcαSer/Thr
Epitope	
Blood groups O, H	Fucα1-2Gal-
Blood group A	GalNAcα1-3(Fucα1-2)Gal-
Blood group B	Galα1-3(Fucα1-2) Gal-
Linear B	Galα1-3Gal-
Blood group i	Galβ1-4GlcNAcβ1-3Gal-
Blood group I	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ13) Gal-
Blood group Sd(a), Cad	GalNAcβ1-4(Siaα2-3)Gal-
Blood group Lewisa	Galβ1-3(Fucα1-4)GlcNAc-
Blood group Lewisx	Galβ1-4(Fucα1-3)GlcNAc-

5.2 Analysis of O-linked Glycosylation

For a while now, O-linked glycosylation has been overlooked by researchers due to a variety of reasons. Firstly, O-glycosylation lacks a known amino acid consensus sequence. In contrast to N-linked glycosylation, the sites for O- linked glycosylation are not found in any known amino acid sequence making it difficult for the development of any tools that can be used as predictors (Jensen et al. 2009). Secondly, the analysis of O-glycans remain a challenge because of the structural heterogeneity of mucins. This has made system wide analysis of mucin difficult because there is no universal enzyme for the removal of glycans from mucins (lwase et al. 1993). Furthermore, O-type glycosylation of mucin is heterogenous thus there is no general isolation and detection method. There has been many attempts to use tags on glycans and although this has been successful, it has been limited to cell culture and animal studies (Agard et al. 2006; Hang et al. 2003). In an attempt to identify the different approaches of characterizing glycoproteins, Dodds et al. (2009) divided the field of glycosylation into three parts namely the proteocentric, glycocentric and reductionist glycoproteomic. The branch of proteocentric deals with the removal of glycans to identify the underlying proteins while glycocentric involves the analysis of glycans removed from a subset of proteins and reductionist glycoproteomic analyses both the glycans and proteins (Dodds et al. 2009). However, due to the complexity of glycoproteins, the authors stressed the need of developing real global glycoproteomic analysing tools that can differentiate and characterize both N and O-linked glycosylation.

5.3 Alterations in Mucin Glycosylation and The Role of Glycosylation in HIV Inhibition.

Alterations and regulation in mucin glycosylation has been associated with certain diseases. For example, the under-glycosylation of mucins has resulted in the formation of cancer associated antigens that are expressed in most carcinomas (Iwata et al. 1993; Vinall et al. 1998; Fowler, Vinall, and Swallow 2001). It has been shown that during oncogenic transformation, mucins tend to be susceptible to aberrant glycosylation patterns. This has been recognized as one of the hallmarks of cancer (Tuccillo et al. 2014). The highly polymorphic nature of mucin genes as a result of the tandem repeats which are variable in number makes them susceptible to alterations in the apomucin core and in the number of O-linked sites that can be available for glycosylation (Vinall et al. 1998; Fowler, Vinall, and Swallow 2001). In addition, mutations that directly affect O-linked glycosylation, either by changing the glycosylation sites or inhibiting the glycosylation processes has been associated with familial tumoral calcinosis, Tn syndrome, IgA neuropathy and thrombocytopenia among other diseases (Jensen et al. 2009). While there is a lot of research showing the relationship between mucins and disease progression especially in the field of cancer, the above shows that mucin glycans can be altered in structure and subsequently affect function.

It is therefore plausible to think that infection with HIV can alter mucin glycans which subsequently compromise their binding ability to HIV. Habte et al. (2010) showed that purified salivary mucins from HIV negative individuals inhibited HIV-1 infection *in vitro* but this inhibitory activity was not observed from individuals who were HIV positive and had different CD4 counts. He postulated that there could be an alteration in the glycosylation pattern of mucins in patients that are HIV positive which could result in the in-ability to inhibit the virus by aggregation. Interestingly, these results contradicted the findings of Peacocke et al. (2012) in which she demonstrated that salivary mucins from both HIV negative and positive individuals inhibited HIV *in vitro*. Furthermore, a study conducted in our laboratory showed that taking into account inter-individual variation, salivary mucins from both HIV negative and positive individuals inhibited HIV-1 activity

in vitro and there was no significant difference in their inhibitory potential towards HIV-1 (McLeod 2017).

During infection, the envelope spike (gp120) binds to the host CD4 co-receptors (CCR5 or CXCR4) and activates the trimerization process in the endoplasmic reticulum (ER). This trimerization process results in protein fold called gp160 which has ten disulphides added to it and approximately 30 N-linked glycans depending on the viral isolate (Land et al. 2003). It is important to note that (HIV-1) envelope glycoprotein is synthesized as a precursor glycoprotein, gp160 (Koga et al. 1994). Thereafter, the cellular enzyme, protease cleaves the gp160 into gp120 and a trans membrane subunit gp41 (Zhu et al. 2000). The N glycosylated region of the gp120 is the site where viral binding to the cell surface receptors occurs suggesting a possible role of the sugar glycans in non-covalent viral interactions (Zhu et al. 2000).

The N-glycosylation of the viral capsid protein has been area of much research (Zhu et al. 2000: Pantophlet et al. 2003). The outer domain of the gp120 has oligo-mannose glycans are highly glycosylated (Zhu et al. 2000) Furthermore, mass spectrometry analysis of viral proteins showed highly conserved virus specific glycan profiles across different primary isolates of geographically divergent clades (Doores et al. 2010). In addition, the glycosylation pattern of HIV was shown to be uniquely conserved across all isolates of HIV -1 from clade A,B and C (Doores et al. 2010). Thus, there is a possibility that mucin glycans can physically interact with the uniquely conserved glycosylation pattern of the envelope gp120 protein of HIV-1 virus thereby trapping the virus and preventing infection of the virus.

Wu et al. (2003) demonstrated that salivary agglutinin, specifically inhibited the infection of HIV-1 by binding to the viral envelope protein gp120. This resulted in no infection of the virus to the host target cells. Furthermore, electron studies conducted by Malamud et al (1993) showed that whole saliva aggregated the HIV virus in a 0.45micro pore size nitrocellulose filter membrane and proposed this as the reason why oral transmission of HIV is rare (Malamud 1993). Therefore, it has been postulated that the role of salivary glycans in the inhibition of HIV infection lies in physically aggregating the

virus. This interaction could possibly occur between the mucin glycans and the sugar sequence of the viral capsid proteins

5.4 Rationale and Aim

This study aims to investigate the role of salivary glycans in-order to gain some insights into the mechanism through which mucins inhibits HIV-1 *in vitro.* By removing the sugar glycans, we aim to see if having a certain number of sugar residues or a specific arrangement of the residues is necessary to have an effect on the anti-HIV-1 activity.

5.5 MATERIALS AND METHODS

5.5.1 Materials

The materials used for this section are described in chapter 2 with a few additions. The enzymatic de-glycosylation kit was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). This enzymes in this kit contained PNGase F, α(2 ,6)Neuramidase, O-Glycosidase, β(1,4) Galactosidase and β-N-Acetylglucosaminidase. TRITON X-100 together with sodium phosphate was purchased from Kimix Chemical and Laboratory Suppliers (Cape Town, South Africa). Bovine fetuin was purchased from Sigma-Aldrich (St. Louis, Missouri, USA).

5.5. 2 Sample Collection

The method for collecting saliva was carried out as described in Chapter 3 with no modifications.

5.5.3 Purification of Salivary Mucins

The purification of salivary mucins was conducted using the methods described in chapter 3.

5.5.4 Sample Preparation.

De-sialylation.

The protein backbone of mucins consists of carbohydrate side chains which are O-linked to serine or threonine residues. The terminal residues on these oligosaccharides are generally N-acetylneuraminic acid (sialic acids). During this process, Proteomics Grade Neuraminidase, a highly purified enzyme from *Arthrobacter ureafaciens,* was used to cleave sialic acid residues from the mucin glycoproteins. It releases α(2→3), α(2→6), α(2→8) and α(2→9) linked sialic acids (Sigma-Aldrich).

Briefly, 1.7 mg of purified freeze-dried mucins were dissolved in 1 ml of 1x Reaction buffer (50 mM sodium phosphate, pH 6.0). Thereafter 4 µl of the neuraminidase enzyme was added to the purified samples and incubated at 37°C for 3 hours. The reaction was stopped by heating at 100°C for 5 minutes. Thereafter, the samples were centrifuged briefly, and the supernatant was collected and dialysed against three changes of water and freeze dried in preparation for de-glycosylation.

De-glycosylation and harvesting of the polypeptide.

Mucins are major structural glycoproteins which are characterized by their high density and high carbohydrate content (Corfield et al. 2001). To investigate the functional significance of the carbohydrate portion of mucins, we used an enzymatic protein de-glycosylation kit (EDEGLY) purchased from Sigma Aldrich (UK) to remove the O-linked glycoproteins. The EDEGLY kit contains enzymes such as PNGase F, β (1→4) Galactosidase, O-Glycosidase, Neuraminidase solution and β-N- Acetylglucosamines whose function is associated with the cleavage of O-linked core glycans on glycoproteins.

De-glycosylation procedure was conducted under native and denaturing conditions to access which conditions would be optimal for the removal of mucin glycans. During denaturing conditions, 100 µg of purified mucin glycoprotein was dissolved in 30 µl of deionized water in an Eppendorf tube. This was followed by the addition of 10 µl of 5x reaction buffer (Triton x100) and 2.5 µl of denaturing solution. The solution was mixed and heated for 5 minutes at 100 ^{0}C . Thereafter, 2.5 µl of Triton x100 was added to increase the activity of the enzymes and 1 µl of PNGase F, O-Glycosidase, α(2,3,6,8,9) Neuramidase solution, 1 µl each of β (1→4) Galactosidase and β-N- Acetyl-glycosaminidase. The tube was incubated for 3 hours at 37°C.

Under native conditions , 100µg of purified freeze dried purified mucin were dissolved in 35µl deionised water in a Eppendorf tube. To this reaction, 10µg of 5X reaction buffer (Triton x100) was added. 1µg of PNGase F, β (1→4) Galactosidase-Glycosidase, Neuraminidase solution and β-N- Acetylglucosaminidase each was then added to the reaction tube. The tube was incubated for 72 hours at 37 °C to allow optimum de-glycosylation to occur. Thereafter, recovery of the polypeptide from the reaction products

was done using the size exclusion gel filtration method (section 2.4.4). This was followed by dialysis against two changes of ammonium bicarbonate (NH_4HCO_3) buffer over a period of 16 hours. The recovered polypeptide was freeze dried and the extent at which glycosylation had occurred was accessed by mobility shifts on an SDS-PAGE gel. The de-glycosylated peptide sample was stored at -20°C for HIV neutralisation assays.

5.5.5 Identification of Mucins

Western Blotting

The presence of MUC5B and MUC7 was determined using the Western blotting method. After SDS Page electrophoresis, proteins and mucins were electroblotted onto a wet nitrocellulose membrane that had been soaked in for an hour in transfer buffer (25mM Tris Base, 150mM Glycine, 10% Methanol at pH 8.3). Thereafter, three layers of filter paper were soaked in the transfer solution and were placed underneath the nitrocellulose membrane followed by the gradient gel and another three filter papers on top of the gel. Mucins were then transferred by vacuum blotting for 1 hour at 40V as described by Mall et al. (2007).

Following this, the nitrocellulose membrane was washed for twenty minutes with Tris buffered saline (TBS) buffer. In-order to prevent any non-specific binding, the membranes were blocked with 5% (w/v) low fat milk in TBS buffer with Tween (TBST), (20mM Tris chloride, 500mM NaCl, 0.05% (v/v) tween 20) for 1 hour. Thereafter, the membranes were washed in TBS buffer for ten minutes and followed by incubation with the appropriate primary antibody(Table 4) which was diluted in in 5% (w/v) low fat milk in TBST. The membranes were then washed twice in TBST buffer followed by incubation with alkaline phosphatase conjugated secondary antibody which diluted in 5% (w/v) low-fat milk powder in 1× TBST for thirty minutes at room temperature. An anti-rabbit IgG alkaline specific antibody secondary antibody was used for both MUC5B and MUC7 with a dilution factor of (1:50 000) (Table 4). After incubation, the membranes were washed three times with TBST for ten minutes. The color was then developed by incubating the membranes with half tablet of NBT/BCIP was dissolved in 5 ml of phosphate-buffered saline (PBS) to give a final concentration of 0.175mg/ml.

Table 8 The primary and secondary antibodies used during western blotting

Primary Antibody	**Manufacturer**	**Type of Antibody**	**Dilution Factor**
MUC5B	Dallas Swallow	Rabbit Monoclonal	1:2000
MUC7	Santa Cruz Biotechnology	Rabbit Polyclonal	1:400
Secondary Antibody	**Manufacturer**	**Type of Antibody**	**Dilution Factor**
Anti-rabbit IgG alkaline specific antibody	Sigma-Aldrich	Goat polyclonal	1:50000

5.5.6 Confirmation of mucin size and purity

The confirmation of mucin size and purity was done as described in Chapter 3 using SDS PAGE analysis followed by PAS staining.

5.5.7 HIV Neutralization assays

The anti-HIV activity of deglycosylated MUC5B and MUC7 was tested against the DU422 and the YU2 pseudo virus using the neutralization assay described in chapter 3 (Montefiori 2009; Sarzotti-Kelsoe et al. 2014).

5.5.8. Cell viability assay

The effect of deglycosylated MUC7 and MUC5B on the viability of TZM-bl/JC cells was tested using the MTT assay as described in chapter 3. The only modification to this method was the use of acidified isopropanol instead of 10% SDS with 0.01M HCl as the solubilizing agent for the formazan crystals.

5.5.9 Data Analysis

We used the methods described in chapter 3 for the processing of the data.

5.6 RESULTS

5.6.1 Confirmation of Mucin Purification and Identity.

The purification of salivary mucins was in line with the representative results t shown in Chapter 4. These samples demonstrated similar protein and glycoprotein profiles after CsCl density gradient ultracentrifugation and Sepharose CL-4B gel filtration. Thereafter, the identity of the V_0 and V_i fractions were investigated by probing the samples against MUC5B and MUC7 antibodies respectively. The V_o stained positive for MUC5B (Figure 23) while the V_i stained positive for MUC7 (Figure 24). Strong staining signal was observed for both MUC5B and MUC7. However, MUC5B showed low electrophoretic mobility on the western blot a characteristic of the highly glycosylated mucin (Wu et al. 1994). MUC7 band was present at approximately 180 kDa and showed more mobility compared to MUC5B (Figure 24). Furthermore, we tested our samples for co-purification a problem demonstrated by Thornton et al. (2001) using slot blot analysis. Our results showed no immunoreactivity when MUC5B samples were probed with anti-MUC7 primary antibody and when MUC7 samples were probed with anti-MUC5B primary antibody (data not shown).

As in Chapter 4, MUC5B samples showed a darker PAS staining and it retained at the top of the running gel during SDS page analysis (Figure 25a). MUC7 appeared as a smear on the 10% gradient gel demonstrating heterogeneity of mucin populations (Figure 25b). The retention of MUC5B at the top of the running gel made it difficult to determine its exact molecular weight. MUC7 showed a band just above 180kDa and the PAS staining was less intense as compared to MUC5B (Figure 25b)

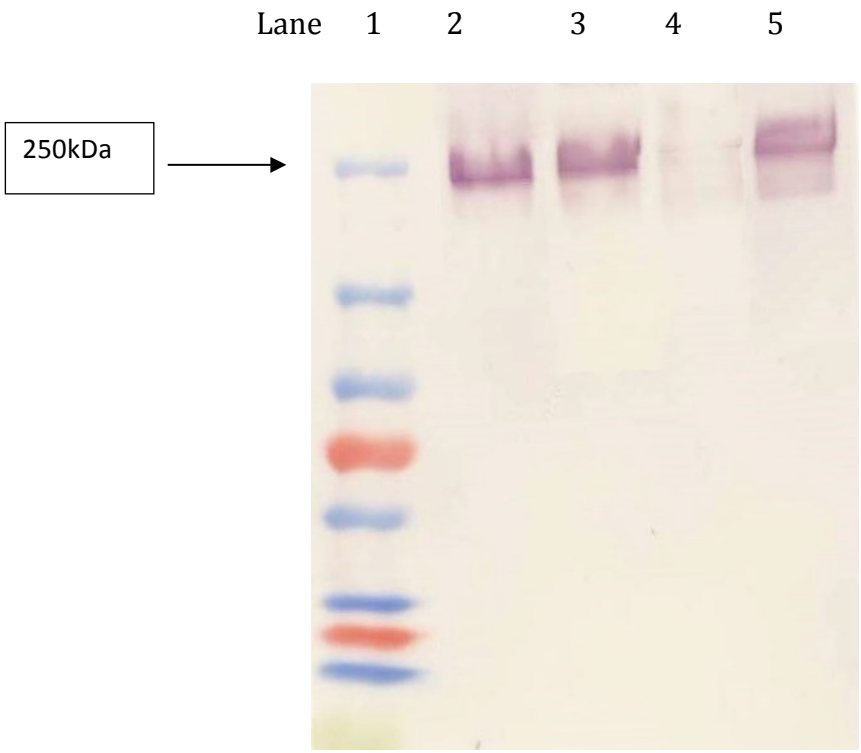


Figure 23 Investigating the identity of four freeze dried Vo fractions after CsCl density gradient ultracentrifugation spin and Sepharose CL-4B gel filtration. The fractions were first subjected to 4-20% SDS-PAGE analysis and stained with PAS staining. Thereafter, a Western blot analysis was performed for MUC5B. Lane 1 is the molecular while lane 2-5 are the V_0 fractions. The arrow indicates the area of interest.

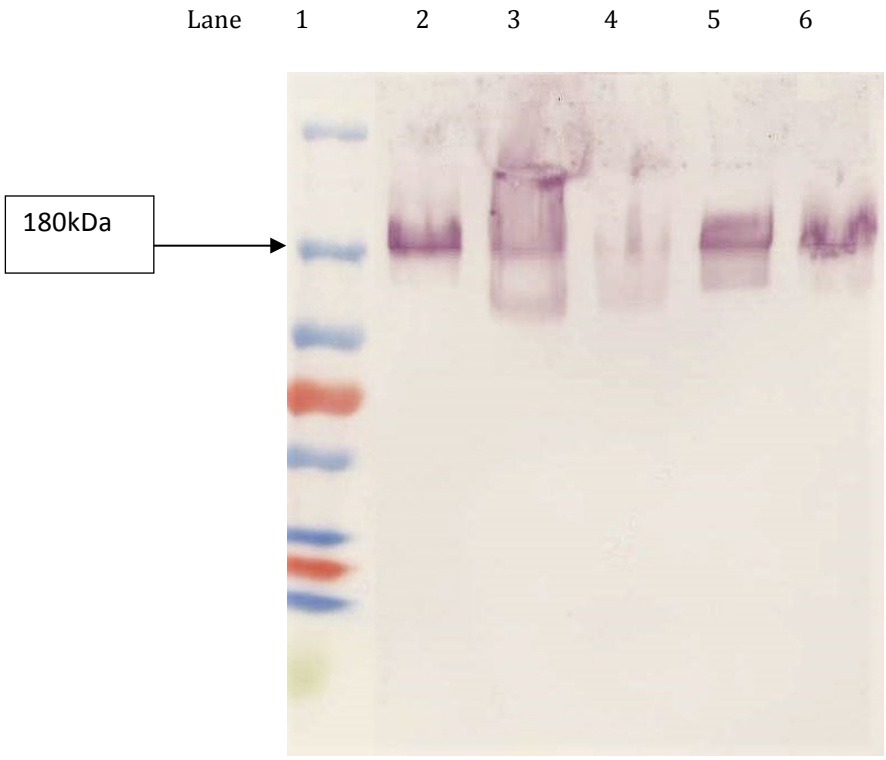


Figure 24 Investigating the identity of four freeze dried V_i fractions after CsCl density gradient ultracentrifugation spin and Sepharose CL-4B gel filtration. The fractions were first subjected to 4-20% SDS-PAGE analysis and stained with PAS staining. Thereafter, a Western blot analysis was performed for MUC7. Lane 1 is the molecular while lane 2-6 are the V_i fractions. The arrow indicates the area ofinterest.

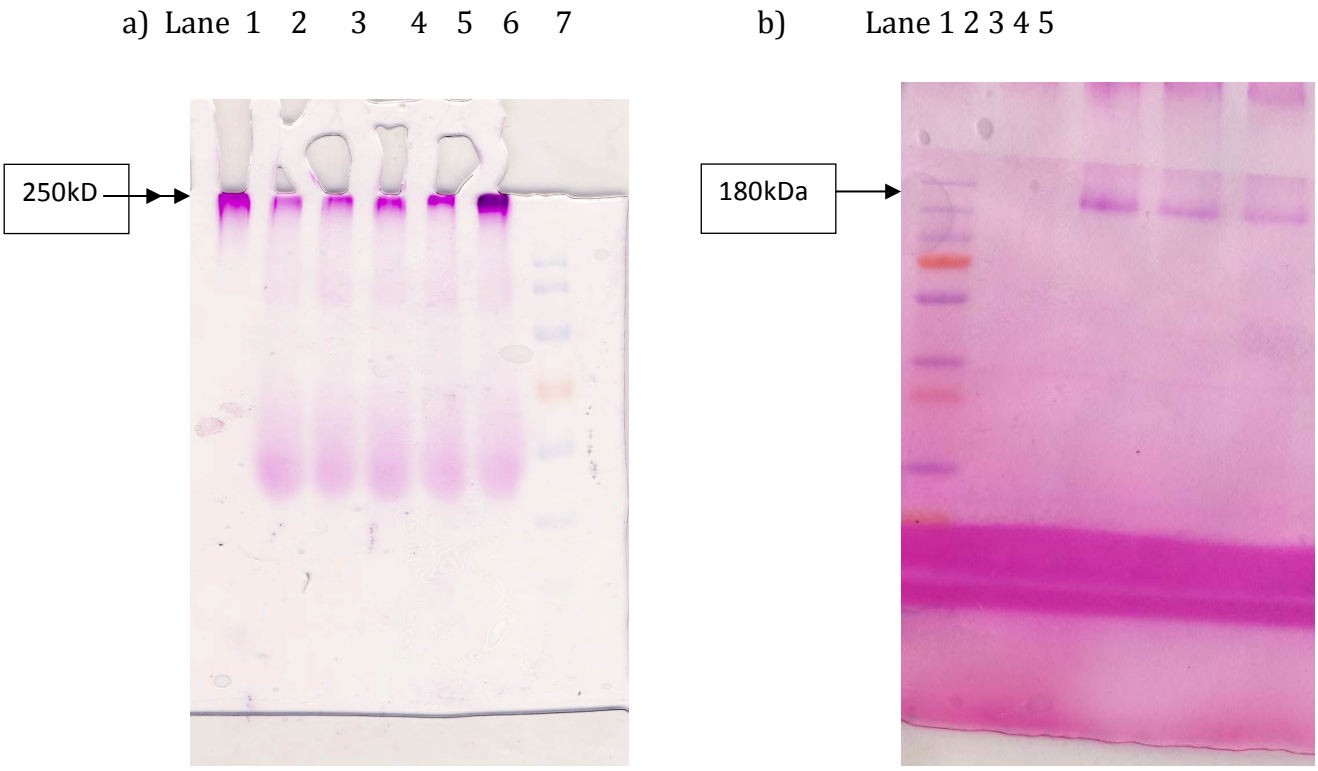


Figure 25 Estimation of mucin size and purity of a) V_0 fractions and b) V_i fractions on a 4-10 % gradient gel SDS-PAGE followed by PAS staining of mucin glycoprotein. Briefly, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation. Lane 7 for a) and Lane 1 for b) represents 10µl of Thermo Scientific Page Ruler Pre-stained Protein Ladder while a) Lane 1-6 shows 10 µl of V_0 b) Lane 2-5 shows 10 µl of V_i fraction. The arrow represents the area of interest.

5.6.2 Confirmation of Mucin De-glycosylation

Purified samples of MUC5B and MUC7 samples were deglycosylated under denaturing and native conditions to assess which conditions gave optimal removal of glycans. Under denaturing conditions, mucins underwent enzymatic de-glycosylation for three hours while under native conditions the process took 72 hours (3 days).

Using the denaturing method, MUC5B and MUC7 (Figure 26 & 27) appeared as smears after three hours demonstrating the heterogeneity of mucin populations (Harding 1984). PAS staining of both samples after three hours showed an intense band. However, both mucin samples showed low mobility in the gradient gel after three hours perhaps due to the extensive carbohydrates side chains that were still attached to the mucins (Figure 26 & 27). This correlated with the intense PAS staining observed. In addition, the removal of mucin glycans after 72 hours using the native method showed an increase in electrophoretic mobility for both samples suggesting the removal of some glycans. Deglycosylated MUC5B samples under native conditions showed less PAS staining intensity compared to MUC7. Since the removal of sugar glycans is a complex process, the possibility that all the sugars were removed is impossible hence the above observation that MUC5B showed low staining intensity could have been caused by the naked carbohydrate regions were not exposed (Figure 26) (Wu et al. 1994).

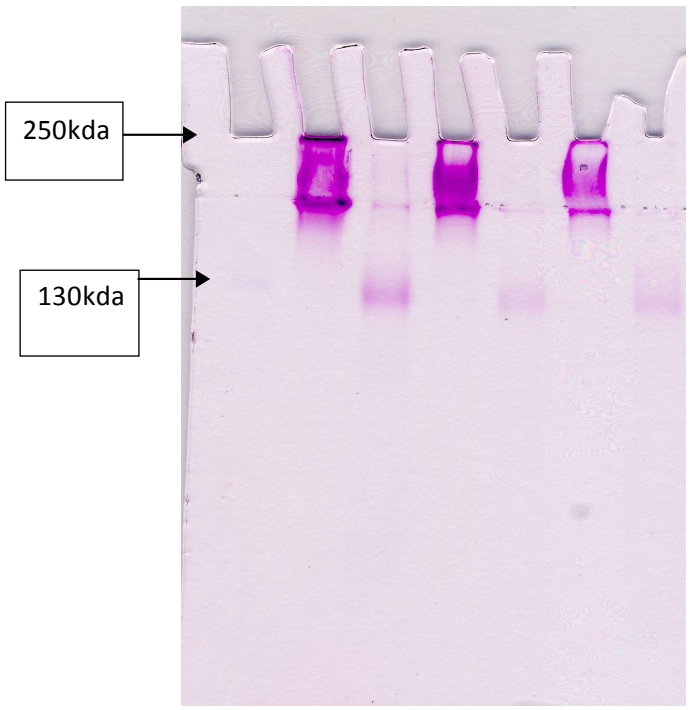


Figure 26 Investigating the enzymatic removal of MUC5B glycans after 3 and 72 hours. Briefly, approximately 100µg of purified freeze-dried purified mucin were dissolved in 35µg deionised water in a Eppendorf tube. To this reaction, 10µg of 5X reaction buffer (Triton x100) was added. 1µg of PNGase F, β (1→4) Galactosidase-Glycosidase, Neuraminidase solution and β-N- Acetylglucosaminidase each was then added to the reaction tube. Thereafter, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation followed by a PAS staining. Lane 1 molecular weight maker, Lane 2, 4, 6 shows MUC5B that was deglycosylated for 3 hours under the denaturing conditions while Lane 3, 5, 7 shows MUC5B sample that was deglycosylated for 72 hours.

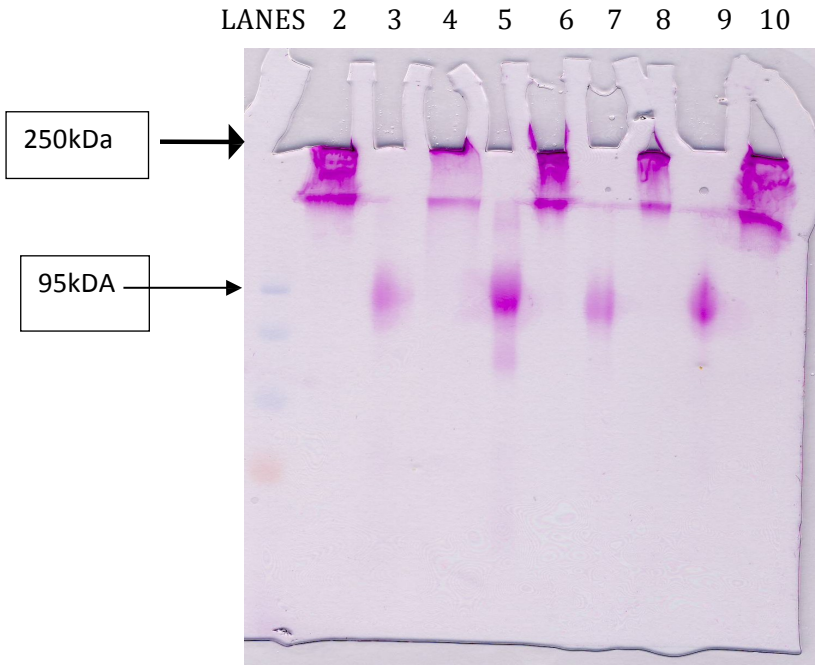


Figure 27 Investigating the enzymatic removal of MUC7 glycans after 3 and 72 hours. Briefly, approximately 100µg of purified freeze dried purified mucin were dissolved in 35µg deionised water in a Eppendorf tube. To this reaction, 10µg of 5X reaction buffer (Triton x100) was added. 1µg of PNGase F, β (1→4) Galactosidase-Glycosidase, Neuraminidase solution and β-N- Acetylglucosaminidase each was then added to the reaction tube. Thereafter, 1mg of purified lyophilized aliquots were dissolved in 100µl of 2% SDS, 1% glycerol and 0.01% bromophenol blue for SDS-PAGE gel investigation followed by a PAS staining. Lane 1 molecular weight maker, Lane 2, 4, 6,8 and 10 shows MUC7 that was deglycosylated for 3 hours under the denaturing conditions while Lane 3, 5, 7 and 9 shows MUC7 sample that was deglycosylated for 72 hours.

5.6.3 HIV-1 Neutralization Assay.

De-glycosylated MUC5B and MUC7

All samples were tested for their ability to inhibit the infection of TZM-bl/JC cells by the YU2 and Du422.1 pseudo virus using the HIV neutralization assay. Samples that displayed no inhibition or that showed no increase in inhibition with an increase in mucin concentration were assigned an arbitrary value of 2000 μg/ml (triple the highest concentration used during these experiments) which was log transformed as it was impossible to extrapolate an IC_{50}. This value did not interfere with our statistical analysis as the data was non-parametric.

The IC_{50} of deglycosylated salivary mucin samples showed high variability between samples that were deglycosylated for three hours vs samples that were deglycosylated for 3 days. The IC_{50} for samples that were deglycosylated for 3 hours ranged between 770ug/ml to 1322ug/ml. Most of the samples that were deglycosylated for 72 hours had IC_{50} that were in the range of 1222ug/ml to1653ug/ml. Only one sample had an IC_{50} that was less than 1000ug/ml (Table 11).

There was a significant difference in the inhibition of the de-glycosylated mucin samples against the DU422 virus (Kruskal-Wallis, p=0.0137)(Figure 28). De-glycosylated MUC5B samples had the highest inhibitory activity against DU422 virus compared to de-glycosylated MUC7 samples (Kruskal-Wallis, p=0.0312). Pairwise analysis of MUC5B and MUC7 after their corresponding incubation time of 3 and 72 hours respectively showed no significant difference in their inhibitory potential against the DU422 virus (Mann-Whitney U, p=0.1836) for MUC5B and (Mann-Whitney U, p=0.0753) for MUC7 samples.

Interestingly, when purified mucin samples were tested against the YU2 virus, no significant difference in the inhibitory potential of these samples was detected (Kruskal-Wallis, p=0.2258)(Figure 29). Pairwise analysis showed that deglycosylated MUC5B sample had a greater inhibitory potential (Mann-Whitney U, p=0.016) against the YU2 virus as compared to the deglycosylated MUC7 sample at both 3 and 72 hours (Mann-Whitney U, p=0.0816) and (Mann-Whitney U, p=0.0636) respectively.

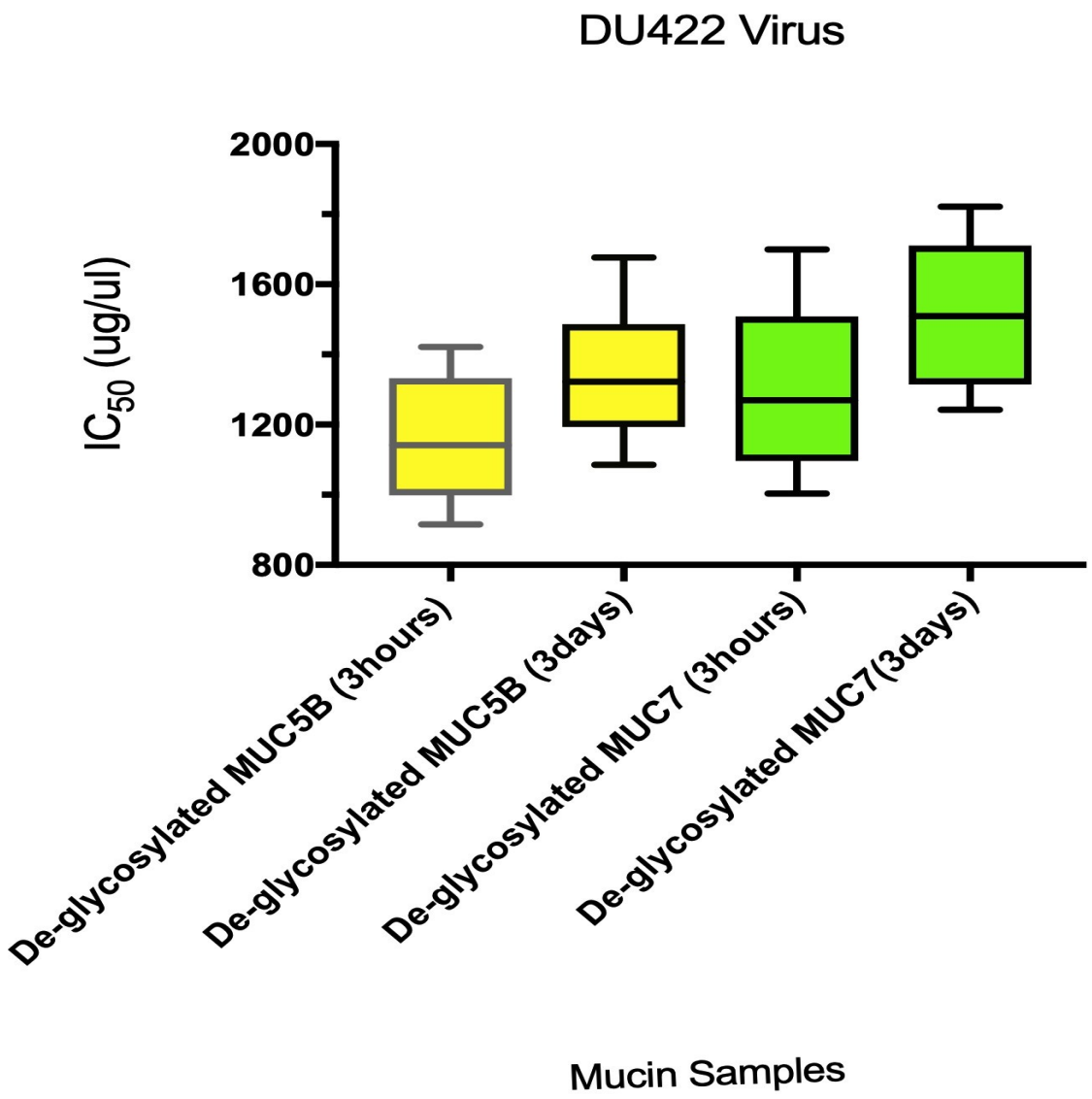


Figure 28 Comparison of the anti-HIV activity of deglycosylated MUC5B and MUC7 samples at different time intervals against the DU422 pseudo virus (Kruskal-Wallis, p=0.0137). De-glycosylated MUC5B samples had the highest inhibitory activity against DU422 virus compared to de-glycosylated MUC7 samples (Mann-Whitney, p=0.0312). Pairwise analysis of MUC5B and MUC7 after their corresponding incubation time of 3 and 72 hours showed no significant difference in their inhibitory potential against the DU422 virus (Mann-Whitney U, p=0.1836) for MUC5B and (Mann-Whitney U, p=0.0753) for MUC7 samples.

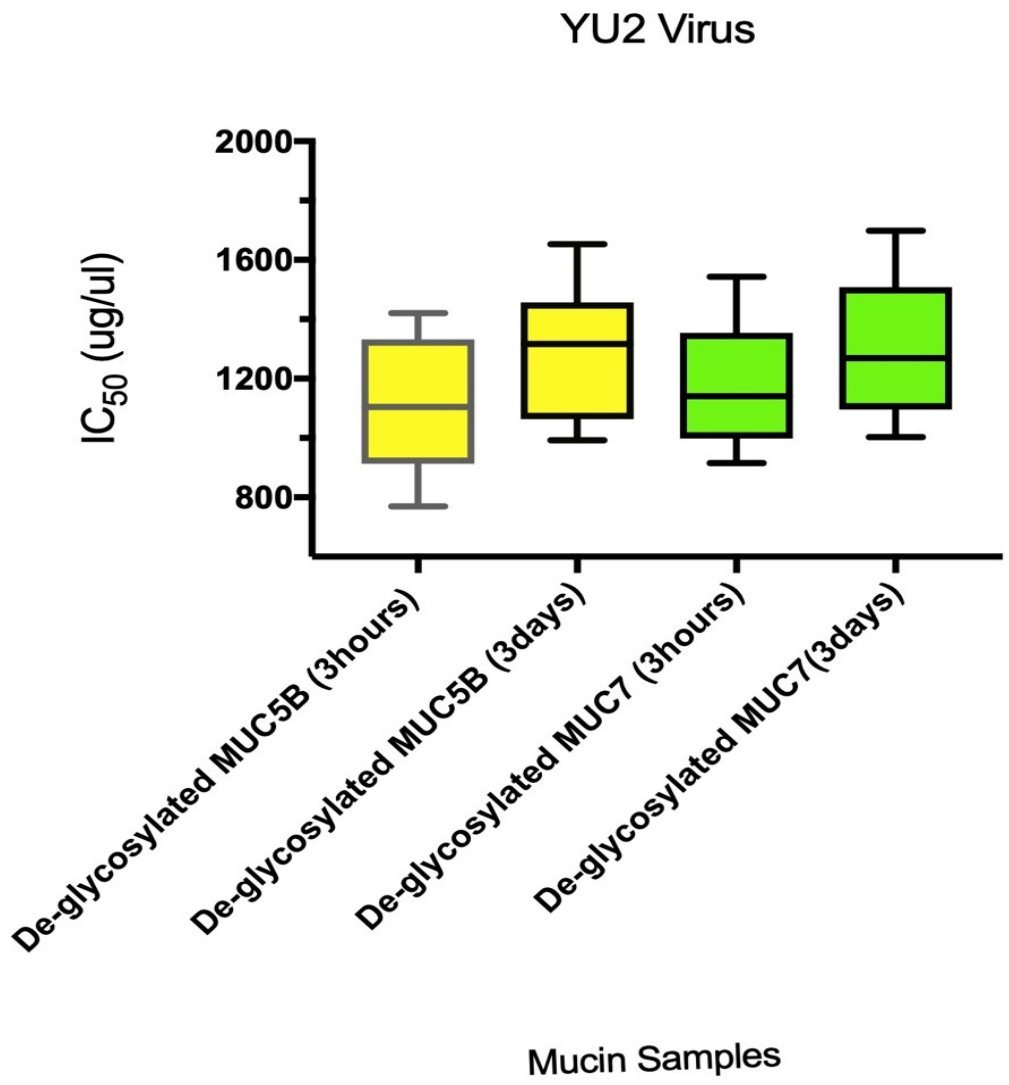


Figure 29 Comparison of the anti-HIV activity of deglycosylated MUC5B and MUC7 samples at different time intervals against the YU2 pseudo virus (Kruskal-Wallis, p=0.2258). Pairwise analysis showed that MUC5B deglycosylated sample for 3 and 72 hours had a greater inhibitory potential (Mann-Whitney U, p=0.016) as compared to the deglycosylated MUC7 sample after the same time intervals (Mann-Whitney U, p=0.0816) and (Mann-Whitney U, p=0.0636) respectively.

5.6.4 MTT Assay for Cell Viability

The cytotoxicity of deglycosylated MUC5B and MUC7 was tested using the MTT assay. As previously described in chapter 4, if the cell viability remained above 70% and there was no increase in cell death with increase in sample concentration, mucins were considered non-toxic. In cases where the above condition was not met, the LC50 (50% Lethal dose) was calculated using the variable slope curve fit function in GraphPad Prism (La Jolla, CA, USA) which calculates the log(inhibitor) vs. normalized response. We also established that in some cases, the amount of virus was the limiting factor for luciferase production.

No detectable cytotoxicity was detected in half of the deglycosylated samples. The remaining deglycosylated samples had LC_{50} that ranged between 668µg/ml to 1738µg/ml. (Table 3, 3.1, 4 & 4.1 in appendix). Some samples showed an increase in cell death as the concentration of the mucin samples increased but no reduction in cell viability over 50% was observed.

Table 9 Cytotoxicity (LC50) and HIV-1 neutralization values of DU422 virus (IC50) by de-glycosylated MUC5B for 3 hours and 72 hours.

	MUC5B 3 hours		MUC5B 3 days	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1099	No toxicity	1221
2	No toxicity	1321	No toxicity	2000
3	No toxicity	1121	No toxicity	1653
4	No toxicity	2000	No toxicity	1085
5	No toxicity	1332	1209	2000
6	No toxicity	1421	567	1421
7	No toxicity	1000	No toxicity	1431
8	758	1332	No toxicity	1332
9	No toxicity	1161	No toxicity	1111

Table 10 Cytotoxicity (LC50) and HIV-1 neutralization values of DU422 virus (IC50) by de-glycosylated MUC7 for 3 hours and 72 hours.

	MUC7 3 hours		MUC7 3 days	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1576	No toxicity	1821
2	No toxicity	1486	No toxicity	1358
3	No toxicity	119 0	No toxicity	1677
4	No toxicity	1087	No toxicity	1242
5	No toxicity	2000	1209	1696
6	No toxicity	1467	567	1256
7	No toxicity	1306	No toxicity	1476
8	758	1003	No toxicity	1753
9	No toxicity	1699	No toxicity	1334

Table 11 Cytotoxicity (LC50) and HIV-1 neutralization values of YU2 virus (IC50) by de-glycosylated MUC7 for 3 hours and 72 hours.

MUC5B 3 hours		MUC5B 3 days	
LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
No toxicity	1332	No toxicity	1221
No toxicity	1421	No toxicity	1312
No toxicity	900	No toxicity	1653
No toxicity	1332	No toxicity	1085
No toxicity	917	1209	1566
No toxicity	770	567	992
No toxicity	992	No toxicity	1332
758	1099	No toxicity	1421
No toxicity	1211	No toxicity	1000

Table 12 Cytotoxicity (LC50) and HIV-1 neutralization values of YU2 virus (IC50) by de-glycosylated MUC7 for 3 hours and 72 hours.

	MUC7 3 hours		MUC7 3 days	
Samples	LC50 (µg/ml)	IC50 (µg/ml)	LC50 (µg/ml)	IC50 (µg/ml)
1	No toxicity	1021	No toxicity	1576
2	No toxicity	1161	No toxicity	1486
3	No toxicity	915	No toxicity	1190
4	No toxicity	992	No toxicity	1087
5	No toxicity	1332	1209	1233
6	No toxicity	1421	567	1467
7	No toxicity	1000	No toxicity	1306
8	758	1321	No toxicity	1003
9	No toxicity	1121	No toxicity	1699

5.7 DISCUSSION

Mucin research has primarily focused on investigating the anti-HIV-1 activity of different mucins and demonstrating if this inhibitory activity is retained in crude secretions (Habte et al. 2006; Habte, de Beer, Lotz, Tyler, Kahn, et al. 2008; Habte, de Beer, Lotz, Tyler, Schoeman, et al. 2008; Peacocke et al. 2012; Mthembu et al. 2014). Alterations in mucin glycosylation has been linked with different diseases suggesting a possible link between mucin polymorphisms and susceptibility to infection (Habte et al. 2010; Peacocke et al. 2012; Mthembu et al. 2014). However, the role of mucin glycans in the inhibition of HIV-1 has been overlooked by researchers because of their structural complexity and heterogeneity (lwase et al. 1993).

This study aimed to investigate the role of salivary glycans in the inhibition of HIV-1. The results of this study suggest that mucin glycans play an important role in inhibiting the transmission of HIV-1. De-glycosylated MUC5B showed higher inhibition against both the YU2 and the DU422 pseudo-virus compared to deglycosylated MUC7(Kruskal-Wallis, p=0.0312). Contrary to purified MUC7 which showed a higher inhibition towards the YU2 virus in chapter 4, deglycosylated MUC7 displayed minimal inhibition towards both viruses compared to deglycosylated MUC5B (Mann-Whitney U, p=0.0816).

The methods used for the purification of salivary mucins was successful. Western blot analysis identified the V_0 and the V_i fractions as MUC5B and MUC7 respectively (Figure 23 and 24) Furthermore, these mucins showed difference in mobility. While MUC5B showed a low electrophoretic mobility on the western blot, MUC7 showed more mobility (Figure 24). These results are in agreement with previous work that has been done in our laboratory which noticed a variation in electrophoretic mobility of MUC7 and MUC5B samples that were isolated from saliva (Habte et al. 2010). SDS Page analysis followed by PAS staining retained MUC5B samples at the top of the running gel (Figure 25a). MUC7 appeared as a smear on the 10% gradient gel demonstrating heterogeneity of mucin populations (Figure 25b). The retention of MUC5B at the top of the running gel made it difficult to determine its exact molecular weight. However, this finding supports the fact

that MUC5B is large in size ,highly glycosylated and has extensive carbohydrate side chains (Wu et al. 1994).

The specific mechanism through which mucin glycosylation inhibits the transmission of HIV-1 is still superficial. It is not known whether the specific arrangement of mucin carbohydrate residues is important in mucin interactions with HIV-1, or if the negative charge afforded by sialic acid and sulfated sugars allows binding to viral receptors. It has been postulated the carbohydrate moiety plays an important role in the ability of mucins to bind and aggregate HIV-1. (Habte et al. 2006; Habte, de Beer, Lotz, Tyler, Kahn, et al. 2008; Peacocke et al. 2012; Mthembu et al. 2014). We investigated the role of salivary glycans using enzymes to de-glycosylate the purified MUC5B and MUC7. Our results (Figure 26 & 27) showed that mucin glycans can be removed using enzymes due to the increase in mobility of mucin samples after de-glycosylating the mucin samples for 72 hours (Figure 26 & 27). Furthermore, our results showed that native conditions are optimal glycan removal. However, a limitation with using this method for de-glycosylation is that the exact oligosaccharides that are removed during the de-glycosylation process is not known. Therefore, in-order to gain an insight as to which mucin glycans were removed, a full sugar analysis using the liquid chromatography electrospray ionisation mass spectrometry (LC ESI-MS) method as described by Thornton et al. (2000) needs to be done The high costs involved in conducting such experiments, restricted our laboratory in investigating the structural variants that were removed. However, this should form the basis of future studies.

Deglycosylated MUC5B showed a higher inhibitory activity towards the Du422 virus and the YU2 virus Mann-Whitney U, $p=0.031$ and Mann-Whitney U, $p=0.016$ respectively (Figure 28 & 29). Interestingly, contrary to purified MUC7 which showed a higher inhibition towards the YU2 pseudo-virus in chapter 4, de-glycosylated MUC7 showed minimal inhibition towards both pseudo-viruses (Mann-Whitney U, $p=0.0816$). The higher inhibition display by de-glycosylated MUC5B suggests the possibility that the extensive oligosaccharides which accounts for 78% of the molecular weight of MUC5B (Levine et al. 1987) are not easily removed. Hence, as mentioned in Chapter 4 , a higher

glycosylation may lend MUC5B the ability to be more effective during viral aggregation. However, the minimal inhibition displayed by deglycosylated mucins as compared to purified mucins, suggests that indeed mucins use glycans to inhibit HIV transmission. Furthermore, the extensive glycosylation of MUC5B prevents the underlying protein core to interact with any viral protein suggesting the possibility that mucin glycans are the sole structures that interact with viral particles (Wu, Csako, and Herp 1994).

Salivary glycans are known to aggregate bacteria and viruses thereby inhibiting viral infectivity into host cells and changes in the pattern of glycosylation and structure of glycans may affect their aggregatory activity. (Bosch et al. 2000; Prakobphol et al. 1999). Interestingly, the glycosylation pattern of salivary mucins particularly MUC5B in females is affected by the menstrual cycle (Prakobphol et al. 1999). It was demonstrated that the expression of MUC5B and the glycosylation pattern is lowest during menstruation and although it increases during the cycle, the levels drops again until the ovulation period ends. In pregnant and lactating woman, the turnover rate of MUC5B glycosylation is demonstrated to be at its peak. Contrary to this, the glycosylation pattern of MUC5B is constant in men and in women who have reached menopause (Prakobphol et al. 1999). Therefore, since glycosylation of MUC5B changes throughout the menstrual cycle, it might be of interest for future studies to check if there is any correlation between the inhibitory activity of MUC5B and the different stages of the menstrual cycle.

This present study shows that deglycosylated MUC5B is significantly more potent in inhibiting the DU422 and the YU2 pseudo-virus compared to de-glycosylated MUC7. Therefore, this suggests that MUC5B can be harnessed and used as a primary constituent of a microbicide that can be used to inhibit the transmission of HIV-1. MUC5B is a major constituent of saliva and under resting conditions, it is produced in higher concentrations as compared to MUC7 (Rayment et al. 2000; Nieuw Amerongen, Bolscher, and Veerman 2004) making it an easier source to use.

CHAPTER SIX: CONCLUSION

According to the report which was published by UNAIDS in 2017, 36.7 million people in the world were infected with HIV and of that total, 1.8 million people were new infections (UNAIDS, 2017). Sub-Saharan Africa was recognised as the most afflicted region worldwide accounting for 26 million people, approximately 68%, living with HIV. In South Africa alone, 7.1 million people were living with HIV in 2017 with approximately 270 000 new infections (UNAIDS, 2017). Kwazulu Natal (KZN), Gauteng and Eastern Cape were reported as provinces with the highest incident rates of HIV in 2016 (National Department of Health 2015). Furthermore, an increase in HIV prevalence usually correlates with an increase in opportunistic infections such as TB increasing the burden of disease (Lawn and Wood 2006, Soeters et al 2005). With the struggle that South Africa is facing in trying to fight this epidemic, effective treatment and preventative strategies are required. This indicates the urgent need for research exploring ways in which HIV transmission can be curbed.

This study aimed to determine the minimum peptide chain length and structure of a gel forming mucin that retains the anti-HIV-1 activity. This is the first time that our laboratory tested crude saliva and purified salivary mucins against a subtype B strain. Furthermore, this study aimed to investigate the role of mucin sugar side chains in the inhibition of HIV-1.

The results of this present study showed that crude saliva, MUC5B and MUC7 are able to inhibit the transmission of HIV DU422.1 and YU2 pseudo-virus strain to TZM-bl/JC cells. While MUC5B showed a higher inhibitory potential against the DU422 virus, MUC7 had a greater anti-HIV-1 activity against the YU2 pseudo-virus strain. Crude saliva showed comparable HIV-1 inhibition against both the DU422 virus and the YU2 virus. In addition, mucins that were reduced and digested by trypsin still retained the anti-HIV-1 activity suggesting that even when the structural conformation of gel forming mucins are compromised, they still retain their function. Interestingly, the de-glycosylation of MUC5B and MUC7 prior to neutralisation assays reduced the anti-HIV activity of these mucins against the YU2 virus and the DU422 pseudo-virus. This indicates the role of mucins glycans in the anti-HIV-1 activity and answers to a certain extent the mechanism through which salivary mucins inhibits HIV-1. However, deglycosylated MUC5B showed

a better inhibitory activity against the YU2 virus and the DU422 virus proving that its extensive glycosylated nature is crucial for inhibition.

The rare transmission of HIV via the oral cavity which is attributed to the innate system, has raised questions on whether salivary components can be used as prophylactics agents, in particular mucins. The limited success of vaccines which involves the adaptive immune response has caused a great increase in research looking at ways which the innate system can protect against HIV-1.

The major constituent of whole human saliva, mucins, have been shown to inhibit HIV-1 *in vitro* by the aggregation of viral particles (Bergey et al. 1994; Wu et al. 2003). While the anti-HIV-1 activity by mucin rich saliva is specific to HIV-1 and not to other envelope viruses such as SIV (Nagashunmugam et al. 1997), its inhibition by purified mucins is not clade or strain specific (Habte et al. 2006; Peacocke 2011; Peacocke et al. 2012). Our results showed that both purified salivary mucins display an inhibitory activity against the subtype B (YU2) strain and the subtype C (DU422) strain, further supporting the above finding.

Furthermore, salivary agglutinin which is the second largest glycoprotein found in saliva has also been shown to inhibit HIV-1 *In vitro* (Wu et al. 2003). A study conducted in our laboratory investigating the anti-HIV activity of SAG and the salivary mucins (MUC5B and MUC7) showed that MUC5B had the highest inhibitory potential as compared to the other two glycoproteins against the DU422 virus (unpublished). Interestingly, our results showed that MUC7 had a great inhibitory potential against the YU2 virus, a subtype B strain. Therefore, by fully understanding how mucins protects the body against HIV-1 entry, we can potentially reduce viral transmission and by doing so, enhance the body's natural innate immune defences. If able to distinguish the key structural variants of mucins which play part in this defence mechanism, we could use this as the core components of a vaginal or rectal microbicide. Thus , MUC5B together with MUC7 must be investigated further to assess if they can be used as core components for a candidate microbicide.

Microbicides provide women with a preventative tool that they can initiate and control. These microbicides are applied to the vagina or the rectum to reduce the transmission of sexually transmitted diseases such as HIV. This is especially important in the Sub-saharan

region where heterosexual transmission is still the major root cause of many HIV related cases. In addition, it is known that women are more highly susceptible to HIV than men. This gender inequality patterns makes women more susceptible to HIV due to the different physiological and biological factors between men and women. The vaginal surface area is a site where many HIV target cells are present, and a place where viral transmission occurs. In addition, the concentration of HIV in semen is higher as compared to vaginal secretions (Ramjee and Daniels 2013). The unreasonably higher risk of infection in women between the ages of 15-24 is due to the underdeveloped genital tract. This delicate tract makes them vulnerable to infections and tissue damage (Ramjee and Daniels 2013).

Apart from biological factors, it is worth mentioning that social factors also play a role in the spread of HIV. Richardson et al. (2014) reported a positive correlation between gender inequality and heterosexual HIV related cases based on the data he obtained from the 2010 UNAIDS Global Report and the 2011 United Nations Human Development (UNDP). Patriarchal culture is still dominant in most African countries including South Africa and it makes it very hard for women to negotiate condom use as they are viewed inferior in society (Ramjee and Daniels 2013). The mindset of masculinity and sexual promiscuity has resulted in the notion of having multiple partners, a theory that has greatly led to the spread of HIV in many African households (Ramjee and Daniels 2013). With this in mind, microbicides can provide women with an HIV preventative tool that they can use without the consent of their partners or in cultural or religious circumstances where condom use is not endorsed.

Several microbicides have been tested but very few have made it to be sold commercially (Abdool Karim and Baxter 2012). Only two microbicides namely 1% tenofovir gel and dapivirine rings have been shown to reduce the risk of HIV acquisition. The tenofovir gel has had some conflicting results from individuals using it. The phase two clinical results showed a 39% efficacy in candidates who used the gel before and after sexual intercourse and 54% reduction in individuals who confirmed consistent adherence (Abdool Karim et al. 2010). Interestingly, the Microbicide Trial Network's VOICE study, Vaginal and Oral Interventions to Control the Epidemic (VOICE) conducted a phase llb clinical study from 2009 till 2011 testing tenofovir and two oral antiretroviral drugs and showed no protective effect against HIV (https://www.clinicaltrials.gov/ct2/show/NCT00705679).

A phase three clinical trial showed no difference in HIV acquisition among the control and placebo group (Rees et al. 2015). Trial results from the monthly use of the dapivirine ring showed that it had the potential to reduce HIV infections by 50% (Baeten et al. 2016). The fact that both microbicides are made from core components of antiretrovirals, raises the possibility of drug resistance if inconsistently used following infection and treatment. This could lead to the formation of drug resistant HIV mutations (Abdool Karim and Baxter 2012).

The absence of any antiretroviral drugs in MUC5B makes it unlikely to induce viral resistance as a prophylactic drug. Furthermore, the gel forming properties of MUC5B together with its rheological properties makes it a good candidate for a microbicide. In addition, the large size and the extensive glycosylation pattern of MUC5B allows for the effective aggregation of HIV-1, giving it higher anti-HIV-1 potency than MUC7. This further solidifies its role as a core component of a vaginal or rectal microbicide. The presence of mucins in the genital tract, makes it impossible for MUC5B to cause any side effects. As demonstrated by our results, even after the removal of mucin glycans, MUC5B still has the highest anti-HIV activity suggesting that it is difficult to compromise the structural abilities of this mucin.

The ability of HIV-1 to evade the immune system during initial infection, together with the presence of viral strains that are resistant to antiretrovirals makes it difficult to completely eradicate this virus. While ARVs provide sufficient protection to boost the immune system, they require strict adherence and managing the side effects is usually difficult. Therefore, the best way of controlling the HIV epidemic is by preventing the transmission of the virus. Currently, the consistent and correct use of condoms and the dapivirine ring offers a preventative tool that women can use. The innate factors provided by saliva may be beneficial in providing a broad neutralization activity against different HIV-1 strains and in eliminating the likelihood of having viral resistance. The results of this study suggest that MUC5B can be harnessed and used as an innate inhibitory factor which can prevent HIV transmission. The presence of glycans may be important for this anti-HIV-1 inhibitory activity.

References

1. Abbasi, J. 2017. “Large HIV Vaccine Trial Launches in South Africa.” The Journal of the American Medical Association 317 (4): 350.
2. Abdool Karim, Q, SS Abdool Karim, JA Frohlich, AC Grobler, C Baxter, LE Mansoor, ABM Kharsany, et al. 2010. “Effectiveness and Safety of Tenofovir Gel, an Antiretroviral Microbicide, for the Prevention of HIV Infection in Women.” Science 329 (5996): 1168–1174.
3. Abdool Karim, SS, and C Baxter. 2012. “Overview of Microbicides for thePrevention of Human Immunodeficiency Virus.” Best Practice & Research Clinical Obstetrics and Gynaecology 26 (4): 427–439.
4. Agard NJ, Baskin JM, Prescher JA, Lo A & BertozziCR (2006) A comparative study of bioorthogonalreactions with azides.ACS Chem Biol1, 644–648.
5. Allen, A. & Snary, D., 1972. The Structure and Function of Gastric Mucus. Gut, 13(8), pp.666–672.
6. Allen, A. 1978. “Structure of Gastrointestinal Mucus Glycoproteins and the Viscous and Gel-Forming Properties of Mucus.” British Medical Bulletin 34 (1): 28–33.
7. Allen, A. 1982. “Structure and Function of Gastrointestinal Mucus.” In Physiology of the Gastrointestinal Tract, edited by LR Johnson, 1st ed, 359–382. New York: Raven Press
8. Allen, A., 1978. Structure of Gastrointestinal Mucus Glycoproteins and the Viscous and Gel-Forming Properties of Mucus. British Medical Bulletin, 34(1), pp.28–33.
9. Allen, A., 1981. Structure and Function of Gastrointestinal Mucus. In L. R. Johnson, ed. New York: Raven Press, pp. 617–639.
10. Bobek, L a, H Tsai, a R Biesbrock, and M J Levine. 1993. “Molecular Cloning, Sequence, and Specificity of Expression of the Gene Encoding the Low Molecular Weight Human Salivary Mucin (MUC7).” The Journal of Biological Chemistry 268 (27): 20563–20569.
11. Bolscher, JG, K Nazmi, LJ Ran, FA van Engelenburg, H Schuitemaker, EC Veerman, and AV Nieuw Amerongen. 2002. “Inhibition of HIV-1 IIIB and Clinical Isolates by Human Parotid, Submandibular, Sublingual and Palatine Saliva.” European Journal of Oral Sciences 110 (2): 149–456.
12. Bolscher, JGM, J Groenink, JS van der Kwaak, PAM van den Keijbus, W van’t Hof, ECI Veerman, and AV Nieuw Amerongen. 1999. “Detection and Quantification of MUC7 in Submandibular, Sublingual, Palatine and Labial Salive by Anti-Peptide Antiserum.” Journal of Dental Research 78 (7): 1362–1369.
13. Bradford, M.., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, pp.248–254.

14. Bradford, MM. 1976. “A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding.” Analytical Biochemistry 72 (1–2): 248–254.
15. Brockhausen, I, H Schachter, and P Stanley. 2009. “O-GalNAc Glycans.” In Essentials of Glycobiology, edited by A Varki, RD Cummings, and JD Esko, 2nd ed. New York: Cold Spring Harbor Laboratory Press.
16. Buisine, M. et al., 2001. Mucin gene expression in intestinal epithelial cells in Crohn’s disease. Gut, 49, pp.544–551.
17. Carlstedt, I, and JK Sheehan. 1984a. “Macromolecular Architecture and Hydrodynamic Properties of Human Cervical Mucins.” Biorheology 21 (1–2): 225–233
18. Carlstedt, I, and JK Sheehan. 1984b. “Macromolecular Properties and Polymeric Structure of Mucus Glycoproteins.” Ciba Founcation Symposium 109: 157–172.
19. Carlstedt, I. & Sheehan, J.K., 1984. Macromolecular properties and polymeric strcture of mucus glycoproteins. In Mucus and Mucosa. pp. 157–172.
20. Chen, J.C.-H. et al., 2000. Crystal structure of the HIV-1 integrase catalytic core and C-terminal domains: A model for viral DNA binding. Proceedings of the National Academy of Sciences, 97(15), pp.8233–8238. Available at: http://www.pnas.org/cgi/doi/10.1073/pnas.150220297.
21. Chen, Y, YH Zhao, TB Kalaslavadi, E Hamati, K Nehrke, AD Le, DK Ann, and R Wu. 2004. “Genome-Wide Search and Identification of a Novel Gel-Forming Mucin MUC19/Muc19 in Glandular Tissues.” American Journal of Respiratory Cell and Molecular Biology 30 (2): 155–165.
22. Chen, Z. et al., 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. Journal of virology, 71(5), pp.3953–60.
23. Chu, Y, J Li, X Wu, Z Hua, and Z Wu. 2013. “Identification of Human Immunodeficiency Virus Type 1 (HIV-1) gp120-Binding Sites on Scavenger Receptor Cysteine Rich 1 (SRCR1) Domain of gp340.” Journal of Biomedical Science 20 (44). doi:10.1186/1423-0127-20-44.
24. Cohen, M.S. et al., 2008. The spread, treatment, and prevention of HIV-1: Evolution of a global pandemic. Journal of Clinical Investigation, 118(4), pp.1244–1254.
25. Collins, K.B. et al., 2000. Development of an in vitro organ culture model to study transmission of HIV-1 in the female genital tract
26. Corfield, AP. 2015. “Mucins: A Biologically Relevant Glycan Barrier in Mucosal Protection.” Biochimica et Biophysica Acta 1850 (1): 236–252.
27. Creeth, J.M. & Denborough, M.A., 1970. The use of equilibrium-density-gradient methods for the preparation and characterization of blood-group-specific glycoproteins. Biochemical Journal, 117(5), pp.879–891.
28. Creeth, JM, and MA Denborough. 1970. “The Use of Equilibrium-Density-Gradient Methods for the Preparation and Characterization of Blood-Group-Specific Glycoproteins.” Biochemical Journal 117 (5): 879–891.
29. Creeth, JM. 1978. “Constituents of Mucus and Their Separation.” British Medical Bulletin 34 (1): 17–24.

30. Desseyn, JL, JP Aubert, I Van Seuningen, N Porchet, and A Laine. 1997. “Genomic Organization of the 3′ Region of the Human Mucin GeneMUC5B.” The Journal of Biological Chemistry 272 (27): 16873–16883.
31. Duriux-Smith, A, and JT Goodman. 1992. “Comparison of Female to Male and Male to Female Transmission of HIV in 563 Stable Couples. European Study Group on Heterosexual Transmission of HIV.” British Medical Journal 304 (6830): 809–813.
32. Duriux-Smith, A. & Goodman, J., 1992. Comparison of Female to Male and Male to Female Transmission of HIV in 563 Stable Couples. European Study Group on Heterosexual Transmission of HIV. British Medical Journal, 304(6830), pp.809–813.
33. Engelman, A, and P Cherepanov. 2012. “The Structural Biology of HIV-1: Mechanistic and Therapeutic Insights.” Nature Reviews Microbiology 10 (4): 280–290.
34. Engelman, A. & Cherepanov, P., 2012. The Structural Biology of HIV-1: Mechanistic and Therapeutic Insights. Nature Reviews Microbiology, 10(4), pp.280–290.
35. Ensoli, B, A Cafaro, P Monini, S Marcotullio, and F Ensoli. 2014. “Challenges in HIV Vaccine Research for Treatment and Prevention.” Frontiers in Immunology 5 (417): 61–75. Fábián, TK, P Hermann, A Beck, P Fejérdy, and G Fábián. 2012. “Salivary Defense Proteins: Their Network and Role in Innate and Acquired Oral Immunity.” International Journal of Molecular Sciences 13 (4): 4295–4320.
36. Finzi, D, J Blankson, JD Siliciano, JB Margolick, K Chadwick, T Pierson, K Smith, et al. 1999. “Latent Infection of CD4+ T Cells Provides a Mechanism for Lifelong Persistence of HIV-1, Even in Patients on Effective Combination Therapy.” Nature Medicine 5 (5): 512–517. 114
37. Fox, PC, A Wolff, CK Yeh, JC Atkinson, and BJ Baum. 1989. “Salivary Inhibition of HIV-1 Infectivity: Functional Properties and Distribution in Men, Women, and Children.” The Journal of the American Dental Association 118 (6): 709–711.
38. Fox, PC, A Wolff, Yeh CK, JC Atkinson, and Baum BJ. 1988. “Saliva Inhibits HIV-1 Infectivity.” The Journal of the American Dental Association 116 (6): 635–637.
39. Frenkel, ES, and K Ribbeck. 2015a. “Salivary Mucins in Host Defense and Disease Prevention.” Journal of Oral Microbiology 7. doi:10.3402/jom.v7.29759.
40. Frenkel, ES, and K Ribbeck. 2015b. “Salivary Mucins Protect Surfaces from Colonization by Cariogenic Bacteria.” Applied and Environmental Microbiology 81 (1): 332–338.
41. Friedman, SR, and DC Des Jarlais. 1991. “HIV among Drug Injectors: The Epidemic and the Response.” AIDS Care 3 (3): 239–250.
42. Fultz, PN. 1986. “Components of Saliva Inactivate Human Immunodeficiency Virus.” Lancet 328 (8517): 1215.
43. Gallo, RC, SZ Salahuddin, M Popovic, GM Shearer, M Kaplan, BF Haynes, TJ Palker, et al. 1984. “Frequent Detection and Isolation of Cytopathic Retroviruses (HTL V -II) from Patients with AIDS and at Risk for AIDS.” Science 224 (4648): 500–503.
44. Gao, F, E Bailes, DL Robertson, Y Chen, CM Rodenburg, SF Michael, LB Cummins, et al. 1999. “Origin of HIV-1 in the Chimpanzee Pan Troglodytes Troglodytes.” Nature 397 (6718): 436–441.
45. Gibbins, HL, GB Proctor, GE Yakubov, S Wilson, and GH Carpenter. 2015. “SIgA Binding to Mucosal Surfaces Is Mediated by Mucin-Mucin Interactions.” PLoS ONES One 10 (3). doi:10.1371/journal.pone.0119677.

46. Gibbons, RJ, and JV Qureshi. 1978. “Selective Binding of Blood Group-Reactive Salivary Mucins by Streptococcus Mutans and Other Oral Organisms.” Infection and Immunity 22 (3): 665–671.
47. Gipson, IK, SB Ho, SJ Spurr-Michaud, AS Tisdale, Q Zhan, E Torlakovic, J Pudney, DJ Anderson, NW Toribara, and JA Hill. 1997. “Mucin Genes Expressed by Human Female Reproductive Tract Epithelia.” Biology of Reproduction 56 (4): 999–1011.
48. Gordon, M, T De Oliveira, K Bishop, HM Coovadia, L Madurai, S Engelbrecht, E Janse van Rensburg, A Mosam, A Smith, and S Cassol. 2003. “Molecular Characteristics of Human Immunodeficiency Virus Type 1 Subtype C Viruses from KwaZulu-Natal, South Africa: Implications for Vaccine and Antiretroviral Control Strategies.” Journal of Virology 77 (4): 2587–2599.
49. Gordon, M. et al., 2003. Molecular Characteristics of Human Immunodeficiency Virus Type 1 Subtype C Viruses from KwaZulu-Natal, South Africa: Implications for Vaccine and Antiretroviral Control Strategies. Journal of Virology, 77(4), pp.2587–2599.
50. Goto, Y, CK Yeh, AL Notkins, and BS Prabhakar. 1991. “Detection of Proviral Sequences in Saliva of Patients Infected with Human Immunodeficiency Virus Type 1.” AIDS Research 116
51. Groot, F, TBH Geijtenbeek, RW Sanders, CE Baldwin, M Sanchez-Hernandez, R Floris, Y Van Kooyk, et al. 2005. “Lactoferrin Prevents Dendritic Cell-Mediated Human Immunodeficiency Virus Type 1 Transmission by Blocking the DC-SIGN--gp120 Interaction.” Journal of Virology 79 (5): 3009–3015.
52. Haase, A.T. 2011. Early events in sexual transmission of HIV and SIV and opportunities for interventions. Annual review of medicine, 62, pp.127–139.
53. Habte, H.H. et al., 2007. Antiviral Activity of Purified Human Breast Milk Mucin. Neonatology, 92, pp.96–104.
54. Habte, H.H. et al., 2008. Inhibition of Human Immunodeficiency Virus Type 1 Activity by Purified Human Breast Milk Mucin (MUC1) in an Inhibition Assay. Neionatology, 93, pp.162–170.
55. Habte, HH, AS Mall, C de Beer, ZE Lotz, and D Kahn. 2006. “The Role of Crude Human Saliva and Purified Salivary MUC5B and MUC7 Mucins in the Inhibition of Human Immunodeficiency Virus Type 1 in an Inhibition Assay.” Virology Journal 3 (99). doi:10.1186/1743-422X-3-99.
56. Habte, HH, C de Beer, ZE Lotz, MG Tyler, D Kahn, and AS Mall. 2008. “Inhibition of Human Immunodeficiency Virus Type 1 Activity by Purified Human Breast Milk Mucin (MUC1) in an Inhibition Assay.” Neonatology 93 (3): 162–170.
57. Habte, HH, C de Beer, ZE Lotz, MG Tyler, L Schoeman, D Kahn, and AS Mall. 2008. “The Inhibition of the Human Immunodeficiency Virus Type 1 Activity by Crude and Purified Human Pregnancy Plug Mucus and Mucins in an Inhibition Assay.” Virology Journal 5 (59). doi:10.1186/1743-422X-5-59.
58. Habte, HH, C de Beer, ZE Lotz, P Roux, and AS Mall. 2010. “Anti-HIV-1 Activity of Salivary MUC5B and MUC7 Mucins from HIV Patients with Different CD4 Counts.” Virology Journal 7 (269). doi:10.1186/1743-422X-7-269.
59. Harmsen, MC, PJ Swart, MP de Béthune, R Pauwels, E De Clercq, TH The, and DK Meijer. 1995. “Antiviral Effects of Plasma and Milk Proteins: Lactoferrin Shows Potent Activity against Both Human Immunodeficiency Virus and Human Cytomegalovirus Replication in Vitro.” The Journal of Infectious Diseases 172 (2): 380–388. 118

60. Ho, DD, T Moudgil, and M Alam. 1989. “Quantitation of Human Immunodeficiency Virus Type 1 in the Blood of Infected Persons.” The New England Journal of Medicine 321 (24): 1621–1625.
61. Hohenester, E, T Sasaki, and R Timpl. 1999. “Crystal Structure of a Scavenger Receptor Cysteine-Rich Domain Sheds Light on an Ancient Superfamily.” Nature Structural & Molecular Biology 6 (3): 228–232.
62. Holmberg, KV, and MP Hoffman. 2014. “Anatomy, Biogenesis, and Regeneration of Salivary Glands.” Saliva: Secretion and Functions 24. doi:10.1159/000358776.
63. Humphrey, SP, and RT Williamson. 2001. “A Review of Saliva: Normal Composition, Flow, and Function.” The Journal of Prosthetic Dentistry 85 (2): 162–169.
64. Ihalin, R, V Loimaranta, and J Tenovuo. 2016. “Origin, Structure, and Biological Activities of Peroxidases in Human Saliva.” Archives of Biochemistry and Biophysics 445 (2): 261–268.
65. Imai, J, M Ghazizadeh, Z Naito, and G Asano. 2001. “Immunohistochemical Expression of T, Tn and Ial-Tn Antigens and Clinical Outcome in Human Breast Carcinoma.” Anticancer Research 21 (2B): 1327–1334.
66. Iwase H, Ishii-Karakasa I, Fujii E, Hotta K, Hiki Y& Kobayashi Y (1992) Analysis of glycoform ofO-glycan from human myeloma immunoglobulin a1by gas-phase hydrazinolysis following pyridyl-amination of oligosaccharides.Anal Biochem206,202–205
67. Jensen, JL, and P Barkvoll. 1998. “Clinical Implications of the Dry Mouth. Oral Mucosal Diseases.” Annals of the New York Academy of Sciences 15 (842): 156–162.
68. Jenssen, H, and REW Hancock. 2009. “Antimicrobial Properties of Lactoferrin.” Biochemie 91 (1): 19–29. 120
69. Kazmi, S.H. et al., 2006. Comparison of human immunodeficiency virus type 1-specific inhibitory activities in saliva and other human mucosal fluids. Clinical and Vaccine Immunology, 13(10), pp.1111–1118.
70. Kazmi, SH, JR Naglik, SP Sweet, RW Evans, S O’Shea, JE Banatvala, and SJ Challacombe. 2006. “Comparison of Human Immunodeficiency Virus Type 1-Specific Inhibitory Activities in Saliva and Other Human Mucosal Fluids.” Clinical and Vaccine Immunology 13 (10): 1111–1118.
71. Kirkbride, HJ, JG Bolscher, K Nazmi, LE Vinall, MW Nash, FM Moss, DM Mitchell, and DM Swallow. 2001. “Genetic Polymorphism of MUC7: Allele Frequencies and Association with Asthma.” European Journal of Human Genetics 9 (5): 347–354.
72. Klasse, P.J., 2012. The molecular basis of HIV entry. Cellular microbiology, 14(8), pp.1183–1192.
73. Konopka, K, N Shine, E Pretzer, and N Düzgüneş. 1999. “Secretory Leukocyte Protease Inhibitor (SLPI): Oxidation of SLPI Does Not Explain Its Variable Anti-HIV Activity.” Journal of Dental Research 78 (12): 1773–1776.
74. Koop, Hm, M Valentijn-Benz, AV Nieuw Amerongen, PA Roukema, and J de Graaff. 1990. “Involvement of Human Mucous Saliva and Salivary Mucins in the Aggregation of the Oral Bacteria Streptococcus Sanguis, Streptococcus Oralis, and Streptococcus Rattus.” Antonie Van Leeuwenhoek 57 (4): 245–252.
75. Kwong, PD, ML Doyle, DJ Casper, C Cicala, SA Leavitt, S Majeed, TD Steenbeke, et al. 2002. “HIV-1 Evades Antibody-Mediated Neutralization through Conformational Masking of Receptor-Binding Sites.” Nature 420 (6916): 678–682.

76. Kwong, PD, R Wyatt, J Robinson, RW Sweet, J Sodroski, and WA Hendrickson. 1998. “Structure of an HIV gp120 Envelope Glycoprotein in Complex with the CD4 Receptor and a Neutralizing Human Antibody.” Nature 393 (6686): 648–659.
77. Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature, 227, pp.680–685.
78. Laemmli, UK. 1970. “Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4.” Nature 227 (5259): 680–685.
79. Levine, MJ, MC Herzberg, MS Levine, SA Ellison, MW Stinson, HC Li, and T van Dyke. 1978. “Specificity of Salivary-Bacterial Interactions: Role of Terminal Sialic Acid Residues in the Interaction of Salivary Glycoproteins with Streptococcus Sanguis and Streptococcus Mutans.” Infection and Immunity 19 (1): 107–115.
80. Levine, MJ, MS Reddy, LA Tabak, RE Loomis, EJ Bergey, PC Jones, RE Cohen, MW Stinson, and I Al-Hashimi. 1987. “Structural Aspects of Salivary Glycoproteins.” Journal of Dental Research 66 (2): 436–441.
81. Levy, J. a, 2009. HIV pathogenesis: 25 years of progress and persistent challenges. AIDS, 23(2), pp.147–160. Available at: http://content.wkhealth.com/linkback/openurl?sid=WKPTLP:landingpage&an=00002030-200901140-00001.
82. Levy, J.A., 1996. Infection by human immunodeficiency virus--CD4 is not enough. The New England Journal of Medicine, 335(20):15(20), pp.1528–1530.
83. Ligtenberg, AJ, EC Veerman, and AV Nieuw Amerongen. 2000. “A Role for Lewis a Antigens on Salivary Agglutinin in Binding to Streptococcus Mutans.” Antonie Van Leeuwenhoek 77 (1): 21–30.
84. Ligtenberg, AJM, E Walgreen-Weterings, EC Veerman, JJ de Soet, J de Graaff, and AV Nieuw Amerongen. 1992. “Influence of Saliva on Aggregation and Adherence of Streptococcus Gordonii HG 222.” Infection and Immunity 60 (9): 3878–3884.
85. Ligtenberg, AJM, NG Karlsson, and ECI Veerman. 2010. “Deleted in Malignant Brain Tumors-1 Protein (DMBT1): A Pattern Recognition Receptor with Multiple Binding Sites.” International Journal of Molecular Sciences 11 (12): 5212–5233.
86. Ligtenberg, TJM, FJ Bikker, J Groenink, I Tornoe, R Leth-larsen, ECI Veerman, AV Nieuw Amerongen, and Uf Holmskov. 2001. “Human Salivary Agglutinin Binds to Lung 123
87. Lihana, R, D Ssemwanga, AI Abimiku, and N Ndembi. 2012. “Update on HIV-1 Diversity in Africa: A Decade in Review.” AIDS Reviews 14 (2): 83–100.
88. Liuzzi, G, A Chirianni, M Clementi, P Bagnarelli, A Valenza, PT Cataldo, and Piazza M. 1996. “Analysis of HIV-1 Load in Blood, Semen and Saliva: Evidence for Different Viral Compartments in a Cross-Sectional and Longitudinal Study.” AIDS 10 (14): 51–56.
89. Malamud, D, and SM Wahl. 2010. “The Mouth: A Gateway or a Trap for HIV?” AIDS 24 (1): 5–16.
90. Malamud, D, C Davis, P Berthold, E Roth, and H Friedman. 1993. “Human Submandibular Saliva Aggregates HIV.” AIDS Research and Human Retroviruses 9 (7): 633–637. 124
91. Malamud, D, T Nagashunmugam, C Davis, S Kennedy, WR Abrams, R Kream, and HM Friedman. 1997. “Inhibition of HIV Infectivity by Human Saliva.” Oral Diseases 3 (S1): 58–63.

92. Mall, A.S. et al., 1988. Gastric mucins: Different size of subunit according to reducing conditions. Biochemical Society Transactions, 16(4), pp.585–586.
93. Mall, A.S., 2008. Analysis of mucins: role in laboratory diagnosis. Journal of Clinical Pathology, 61(9), pp.1018–1024.
94. Mall, AS, DA Hutton, R Coan, LA Sellers, and A Allen. 1988. "Gastric Mucins: Different Size of Subunit according to Reducing Conditions." Biochemical Society Transactions 16 (4): 585–586.
95. Mall, AS, Z Lotz, M Tyler, P Goldberg, J Rodrigues, D Kahn, N Chirwa, and D Govender. 2011. "Immunohistochemical and Biochemical Characterization of Mucin in Pseudomyxoma Peritonei: A Case Study." Case Reports in Gastroenterology 5 (1): 5–16.
96. Mall, AS. 1988. "Gastro-Duodenal Mucin Isolation and Structure." PhD thesis, University of Newcastle upon Tyne.
97. Mall, AS. 2008. "Analysis of Mucins: Role in Laboratory Diagnosis." Journal of Clinical Pathology 61 (9): 1018–1024.
98. Mantle, M, and A Allen. 1978. "A Colorimetric Assay for Glycoproteins Based on the Periodic acid/Schiff Stain." Biochemical Society Transactions 6 (3): 607–609.
99. Matsuda, S, S Oka, M Honda, Y Takebe, and T Takemori. 1993. "Characteristics of IgA 125
100. McNeely, T B, D C Shugars, M Rosendahl, C Tucker, S P Eisenberg, and S M Wahl. 1997. "Inhibition of Human Immunodeficiency Virus Type 1 Infectivity by Secretory Leukocyte Protease Inhibitor Occurs prior to Viral Reverse Transcription." Blood 90 (3): 1141–1149.
101. McNeely, T B, M Dealy, D J Dripps, J M Orenstein, S P Eisenberg, and S M Wahl. 1995. "Secretory Leukocyte Protease Inhibitor: A Human Saliva Protein Exhibiting Anti-Human Immunodeficiency Virus 1 Activity in Vitro." The Journal of Clinical Investigation 96 (1): 456–464.
102. Mehrotra, R, D J Thornton, and J K Sheehan. 1998. "Isolation and Physical Characterization of the MUC7 (MG2) Mucin from Saliva: Evidence for Self-Association." The Biochemical Journal 334 (2): 415–422.
103. Montefiori, D.C., 2009. Measuring HIV Neutralization in a Luciferase Reporter Gene Assay. In V. R. Prasad & G. V Kalpana, eds. HIV Protocols. Totowa, NJ: Humana Press, pp. 395–405.
104. Montefiori, DC, and TG Evans. 1999. "Toward an HIV Type 1 Vaccine That Generates Potent, Broadly Cross-Reactive Neutralizing Antibodies." AIDS Research and Human Retroviruses 15 (8): 689–698.
105. Montefiori, DC. 2009. "Measuring HIV Neutralization in a Luciferase Reporter Gene Assay." In HIV Protocols, edited by VR Prasad and GV Kalpana, 2nd ed., 485:395–405. Humana Press.
106. Mthembu, Y, Z Lotz, M Tyler, C de Beer, J Rodrigues, L Schoeman, and AS Mall. 2014. "Purified Human Breast Milk MUC1 and MUC4 Inhibit Human Immunodeficiency Virus." Neonatology 105 (3): 211–217.
107. Mthembu, Y. et al., 2014. Purified Human Breast Milk MUC1 and MUC4 Inhibit Human Immunodeficiency Virus. Neionatology, 105(211-217).
108. Murray, PA, A Prakobphol, T Lee, CI Hoover, and SJ Fisher. 1992. "Adherence of Oral Streptococci to Salivary Glycoproteins." Infection and Immunity 60 (1): 31–38.

109. Murray, PA, MJ Levine, LA Tabak, and MS Reddy. 1982. “Specificity of Salivary-Bacterial Interactions: II. Evidence for a Lectin on Streptococcus Sanguis with Specificity for a NeuAca2, 3Galβ31, 3GalNAc Sequence.” Biochemical and Biophysical Research 128
110. Nagashunmugam, T, D Malamud, C Davis, WR Abrams, and HM Friedman. 1998. “Human Submandibular Saliva Inhibits Human Immunodeficiency Virus Type 1 Infection by Displacing Envelope Glycoprotein gp120 from the Virus.” The Journal of Infectious Diseases 178 (6): 1635–1641.
111. Nagashunmugam, T, HM Friedman, C Davis, S Kennedy, LT Goldstein, and D Malamud. 1997. “Human Submandibular Saliva Specifically Inhibits HIV Type 1.” AIDS Research and Human Retroviruses 13 (5): 371–376.
112. Narayan, O, and JE Clements. 1989. “Biology and Pathogenesis of Lentiviruses.” Journal of General Virology 70 (7): 1617–1639.
113. Ng, TB, TL Lam, TK Au, XY Ye, and CC Wan. 2001. “Inhibition of Human Immunodeficiency Virus Type 1 Reverse Transcriptase, Protease and Integrase by Bovine Milk Proteins.” Life Sciences 69 (19): 2217–2223.
114. Peacocke, J, Z Lotz, C de Beer, P Roux, and AS Mall. 2012. “The Role of Crude Saliva and Purified Salivary Mucins in the Inhibition of the Human Immunodeficiency Virus Type 1.” Virology Journal 9 (177). doi:10.1186/1743-422X-9-177. Peacocke, J. 2011. “The Role of Crude Salivary Mucus and Its Purified Mucins MUC5B and MUC7 in the Inhibitionn of the Human Immunodeficiency Virus Type 1.” MSc thesis, University of Cape Town. 130
115. Pearson, JP, A Allen, and S Parry. 1981. “A 70000-Molecular-Weight Protein Isolated From Purified Pig Gastric Mucus Glycoprotein by Reduction of Disulphide Bridges and Its Implication in the Polymeric Structure.” Biochemical Journal 197 (1): 155–162.
116. Perez-Vilar, J, JK Sheehan, and SH Randell. 2003. “Making More MUCs.” American Journal of Respiratory Cell and Molecular Biology 28 (3): 267–270.
117. Pettifor, AE, HV Rees, I Kleinschmidt, AE Steffenson, C MacPhail, L Hlongwa-Madikizela, K Vermaake, and NS Padian. 2005. “Young People’s Sexual Health in South Africa: HIV Prevalence and Sexual Behaviors from a Nationally Representative Household Survey.” AIDS 19 (14): 1525–1534.
118. Pigman, W, J Moschera, M Weiss, and G Tettamanti. 1973. “The Occurrence of Repetitive Glycopeptide Sequences in Bovine Submaxillary Glycoprotein.” European Journal of Biochemistry 32 (1): 148–154.
119. Polonis, VR, BK Brown, A Borges, S Zolla-Pazner, DS Dimitrov, M Zhang, SW Barnett, et al. 2008. “Recent Advances in the Characterization of HIV-1 Neutralization Assays for Standardized Evaluation of the Antibody Response to Infection and Vaccination.” Virology 375 (2): 315–320.
120. Porchet, N, P Pigny, MP Buisine, V Debailleul, P Degand, A Laine, and JP Aubert. 1995. “Human Mucin Genes: Genomic Organization and Expression of MUC4, MUC5AC and 131
121. Prakobphol, A, F Xu, VM Hoang, T Larsson, J Bergstrom, I Johansson, L Frängsmyr, et al. 2000. “Salivary Agglutinin, Which Binds Streptococcus Mutans and Helicobacter Pylori, Is the Lung Scavenger Receptor Cysteine-Rich Protein Gp-340.” Journal of Biological Chemistry 275 (51): 39860–39866.

122. Pudney, J, M Oneta, K Mayer, G Seage, and D Anderson. 1992. “Pre-Ejaculatory Fluid as Potential Vector for Sexual Transmission of HIV-1.” Lancet 340 (8833): 1470.
123. Rajarapu, G. 2014. “Genes and Genome of HIV-1.” Journal of Phylogenetics & Evolutionary Biology 2 (1): 1–7.
124. Ramasubbu, N, MS Reddy, EJ Bergey, GG Haraszthy, SD Soni, and MJ Levine. 1991. “Large-Scale Purification and Characterization of the Major Phosphoproteins and Mucins of Human Submandibular-Sublingual Saliva.” Biochemical Journal 280 (2): 341–352.
125. Ramjee, G, and B Daniels. 2013. “Women and HIV in Sub-Saharan Africa.” AIDS Research and Therapy 10 (30). doi:10.1186/1742-6405-10-30.
126. Ramjee, G. & Daniels, B., 2013. Women and HIV in Sub-Saharan Africa. AIDS Research and Therapy, 10, p.30.
127. Ranganathan, K, BVR Reddy, N Kumarasamy, S Solomon, R Viswanathan, and NW Johnson. 2000. “Oral Lesions and Conditions Associated with Human Immunodeficiency Virus Infection in 300 South Indian Patients.” Oral Diseases 6 (3): 152–157.
128. Rayment, SA, B Liul, GD Offner, FG Oppenheim, and RF Troxler. 2000. “Immunoquantification of Human Salivary Mucins MG1 and MG2 in Stimulated Whole Saliva: Factors Influencing Mucin Levels.” Journal of Dental Research 79 (10): 1765–1772.
129. Reddy, MS, LA Bobek, GG Haraszthy, AR Riesbrock, and MJ Levine. 1992. “Structural Features of the Low-Molecular-Mass Human Salivary Mucin.” Biochemical Journal 287 (2): 639–643.
130. Reid, L, and JR Clamp. 1982. “The Biochemical and Histochemical Nomenclature of Mucus.” British Medical Bulletin 34 (1): 5–8. Rose, M.C. & Voynow, J. a, 2006. Respiratory tract mucin genes and mucin glycoproteins in health and disease. Physiological reviews, 86(1), pp.245–278.
131. Rose, MC, and JA Voynow. 2006. “Respiratory Tract Mucin Genes and Mucin Glycoproteins in Health and Disease.” Physiological Reviews 86 (1): 245–278.
132. Rousseau, K, S Kirkham, L Johnson, B Fitzpatrick, M Howard, EJ Adams, DF Rogers, D Knight, Saeland, E, MA de Jong, AA Nabatov, H Kalay, TB Geijtenbeek, and Y van Kooyk. 2009. 133 Rousseau, K. et al., 2008. Proteomic analysis of polymeric salivary mucins: No evidence for MUC19 in human saliva. Biochemical Journal, 413(3), pp.545–552.
133. Saeland, E. et al., 2009. MUC1 in human milk blocks transmission of human immunodeficiency virus from dendritic cells to T cells. Molecular Immunology, 46(11-12), pp.2309–2316.
134. Sarzotti-Kelsoe, M, RT Bailer, E Turk, CL Lin, M Bilska, KM Greene, H Gao, et al. 2014. “Optimization and Validation of the TZM-Bl Assay for Standardized Assessments of Neutralizing Antibodies against HIV-1.” Journal of Immunological Methods 409: 131–146.
135. Sarzotti-Kelsoe, M. et al., 2014. Optimization and validation of the TZM-bl assay for standardized assessments of neutralizing antibodies against HIV-1. Journal of Immunological Methods, 409(919), pp.131–146.
136. Scawen, M, and A Allen. 1977. “The Action of Proteolytic Enzymes on the Glycoprotein from Pig Gastric Mucus.” Biochemical Journal 163 (2): 363–368.

137. Schulz, BL, AJ Sloane, LJ Robinson, SS Prasad, RA Lindner, M Robinson, PT Bye, et al. 2007. “Glycosylation of Sputum Mucins Is Altered in Cystic Fibrosis Patients.” Glycobiology 17 (7): 698–712.
138. Schulz, BL, D Oxley, NH Packer, and NG Karlsson. 2002. “Identification of Two Highly Sialylated Human Tear-Fluid DMBT1 Isoforms: The Major High-Molecular-Mass Glycoproteins in Human Tears.” Biochemical Journal 366 (2): 511–520.
139. Scott, J. 1979. “The Proportional Volume of Mucous Acinar Cells in Normal Human Submandibular Salivary Glands.” Archives of Oral Biology 24 (6): 479–481.
140. Sellers, L. et al., 1988. Mucus Glycoprotein Gels. Role of Glycoprotein Polymeric Structure and Carbohydrate Side-Chains in Gel-Formation. Carbohydrate Research, 178(1), pp.93–110.
141. Tabak, LA, MJ Levine, NK Jain, AR Bryan, RE Cohen, LD Monte, S Zawacki, GH Nancollas, A Slomiany, and BL Slomiany. 1985. “Adsorption of Human Salivary Mucin to Hydroxyapatite.” Archives of Oral Biology 30 (5): 423
142. Tabak, LA. 1990. “Structure and Function of Salivary Mucins.” Oral Biology and Medicine 1 (4): 229–234
143. Ten Hagen KG, Fritz TA & Tabak LA (2003) All in the family: the UDP-GalNAc:polypeptide N-acety-lgalactosaminyltransferases.Glycobiology13, 1R–16R.
144. Thomsson, KA, A Prakobphol, H Leffler, MS Reddy, MJ Levine, SJ Fisher, and GC Hansson. 2002. “The Salivary Mucin MG1 (MUC5B) Carries a Repetoire of Unique Oligosaccharides That Is Large and Diverse.” Glycobiology 12 (1): 1–14.
145. Thomsson, KA, BL Schulz, NH Packer, and NG Karlsson. 2005. “MUC5B Glycosylation in Human Saliva Reflects Blood Group and Secretor Status.” Glycobiology 15 (8): 791–804.
146. Thornton, D.J. et al., 1996. Respiratory mucins: identification of core proteins and glycoforms. Biochemical Journal1, 316, pp.967–975.
147. Thornton, DJ, JR Davies, S Kirkham, A Gautrey, N Khan, PS Richardson, and JK Sheehan. 2001. “Identification of a Nonmucin Glycoprotein (Gp-340) from a Purified Respiratory Mucin Preparation: Evidence for an Association Involving the MUC5B Mucin.” Glycobiology 11 (11): 969–977.
148. Thornton, DJ, N Khan, R Mehrotra, M Howard, E Veerman, NH Packer, and JK Sheehan. 1999. “Salivary Mucin MG1 Is Comprised Almost Entirely of Different Glycosylated Forms of the MUC5B Gene Product.” Glycobiology 9 (3): 293–302.
149. Todds ED, Seipert RR, Clowers BH, German JB &Lebrilla CB (2009) Analytical performance ofimmobilized pronase for glycopeptide footprinting andimplications for surpassing reductionist glycoproteo-mics.J Proteome Res8, 502–512
150. Troxler, RF, I Lontchcva, FG Oppenheim, DP Nunes, and GD Offner. 1997. “Molecular Characterization of a Major High Molecular Weight Mucin from Human Sublingual Gland.” Glycobiology 7 (7): 965–973.
151. UNAIDS, 2013. GLOBAL REPORT: UNAIDS report on the global AIDS epidemic 2013, Available at: www.unaids.org/.../unaids/.../2013/gr2013/UNAIDS_Global_Report_2013.
152. UNAIDS,2017.FactsheetNovember2016,Availableat: http://www.unaids.org/sites/default/files/media_asset/UNAIDS_FactSheet_en.pdf

153. Wahl, A, C Baker, RA Spagnuolo, LW Stamper, GG Fouda, SR Permar, K Hinde, et al. 2015. “Breast Milk of HIV-Positive Mothers Has Potent and Species-Specific in Vivo HIV-Inhibitory Activity.” Journal of Virology 89 (21): 10868–10876.
154. Wahl, A. et al., 2012. Human breast milk and antiretrovirals dramatically reduce oral HIV-1 transmission in BLT humanized mice. PLoS Pathogens, 8(6), pp.1–13.
155. Wahl, A. et al., 2015. Breast milk of HIV-positive non-transmitting mothers has potent and species-specific in vivo HIV inhibitory activity. Journal of Virology, 89(21), pp.10868–10878.
156. Wang, SL, ZT Zhai, J Li, XZ Zhu, H Dong, and YG Zhang. 1998. “Investigation of the Clinical Value of Total Saliva Flow Rates.” Archives of Oral Biology 43 (1): 39–43. 138
157. Wei, G, and LA Bobek. 2004. “In Vitro Synergic Antifungal Effect of MUC7 12-Mer with Histatin-5 12-Mer or Miconazole.” Journal of Antimicrobial Chemotherapy 53 (5): 750–758.
158. Wei, X, JM Decker, H Liu, Z Zhang, RB Arani, JM Kilby, MS Saag, X Wu, GM Shaw, and JC Kappes. 2002. “Emergence of Resistant Human Immunodeficiency Virus Type 1 in Patients Receiving Fusion Inhibitor (T-20) Monotherapy.” Antimicrobial Agents and Chemotherapy 46 (6): 1896–1905.
159. Wei, X, JM Decker, S Wang, H Hui, JC Kappes, X Wu, JF Salazar-Gonzalez, et al. 2003. “Antibody Neutralization and Escape by HIV-1.” Nature 422 (6929): 307–312.
160. Williamson, C, L Morris, MF Maughan, L Ping, SA Dryga, R Thomas, EA Reap, et al. 2003. “Characterization and Selection of HIV-1 Subtype C Isolates for Use in Vaccine Development.” AIDS Research and Human Retroviruses 19 (2): 133–144.
161. Wu, A.M., Csako, G. & Herp, A., 1994. Structure, Biosynthesis, and Function of Salivary Mucins. Molecular and Cellular Biochemistry, 137(1), pp.39–55.
162. Wu, AM, G Csako, and A Herp. 1994. “Structure, Biosynthesis, and Function of Salivary 139
163. Wu, L, and VN KewalRamani. 2006. “Dendritic-Cell Interactions with HIV: Infection and Viral Dissemination.” Nature Reviews Microbiology 6 (11): 859–868.
164. Wu, L. & KewalRamani, N.V., 2006. Dendritic-cell interactions with HIV: infection and viral dissemination. Nature Reviews Immunology, 6(11), pp.859–868.
165. Wu, W, BL Kemp, ML Proctor, AF Gazdar, JD Minna, WK Hong, and L Mao. 1999. “Expression of DMBT1, a Candidate Tumor Suppressor Gene, Is Frequently Lost in Lung Cancer.” Cancer Research 59 (8): 1846–1851.
166. Wu, Z, D Van Ryk, C Davis, WR Abrams, I Chaiken, J Mangnani, and D Malamud. 2003. “Salivary Agglutinin Inhibits HIV Type 1 Infectivity through Interaction with Viral Glycoprotein 120.” AIDS Research and Human Retroviruses 19 (3): 201–209.
167. Wu, Z, E Golub, WR Abrams, and D Malamud. 2004. “gp340 (SAG) Binds to the V3 Sequence of gp120 Important for Chemokine Receptor Interaction.” AIDS Research and Human Retroviruses 20 (6): 600–607.
168. Wu, Z, S Lee, W Abrams, D Weissman, and D Malamud. 2006. “The N-Terminal SRCR-SID Domain of Gp-340 Interacts with HIV Type 1 gp120 Sequences and Inhibits Viral Infection.” AIDS Research and Human Retroviruses 22 (6): 508–515.
169. Yasuda, S, M Iwasaki, S Oka, S Naganawa, T Nakasone, Mitsuo Honda, Tetsutaro Sata, et al. 1998. “Detection of HIV-Gag p24-Specific Antibodies in Sera and Saliva of HIV-1-Infected Adults and in Sera of Infants Born to HIV-1-Infected Mothers.” Microbiology and Immunology 42 (4): 305–311.

170. Yeh, CK, B Handelman, PC Fox, and BJ Baum. 1992. “Further Studies of Salivary Inhibition of HIV-1 Infectivity.” Journal of Acquired Immune Deficiency Syndromes 5 (9): 898–903.
171. Yu, X, Y Yu, B Liu, K Luo, W Kong, P Mao, and X Yu. 2003. “Induction of APOBEC3G Ubiquitination and Degradation by an HIV-1 Vif-Cul5-SCF Complex.” Science 302 (5647): 1056–1060.
172. Zalewska, A, K Zwierz, K Zólkowski, and A Gindzieñski. 2000. “Structure and Biosynthesis of Human Salivary Mucins.” Acta Biochimica Polonica 47 (4): 1067–1079. 140
173. Zuckerman, R.A. et al., 2004. Higher Concentration of HIV RNA in Rectal Mucosa Secretions than in Blood and Seminal Plasma, among Men Who Have Sex with Men, Independent of Antiretroviral Therapy. The Journal of Infectious Diseases, 190(1), pp.156–161.
174. Zuckerman, RA, WL Whittington, CL Celum, TK Collis, AJ Lucchetti, JL Sanchez, JP Hughes, JL Sanchez, and RW Coombs. 2004. “Higher Concentration of HIV RNA in Rectal Mucosa Secretions than in Blood and Seminal Plasma, among Men Who Have Sex with Men, Independent of Antiretroviral Therapy.” The Journal of Infectious Diseases 190 (1): 156–161

Appendix 1: Buffers, Reagents and Solutions

Guanidinium hydrochloride in PBS, pH 6.5, with protease inhibitors

1. 10 mM PBS
    - 0.568 g Na2PO4 in 400 ml dH20
    - 0.960 g NaH2PO4 in 800 ml dH20
    - Add reagent 1 to 2 until pH 6.5 and store at 4°C
2. 4M GuHCl in PBS, pH 6.5
    - 382 g GuHCl in 1 litre of 10 mM PBS, pH 6.5
3. Protease inhibitors
    - 1 mM PMSF (0.174 g in 1 litre)
    - 5 mM NEM (0.626 g in 1 litre)
    - 10 mM EDTA (3.722 g in 1 litre)

Periodic acid Schiff (PAS) assay for glycoproteins

1. Schiff's reagent
    - 10 g pararosanniline chloride dissolved in 1 litre boiling dH2O, with constant stirring
    - Cool solution to 50°C on bench and add 200 ml 1 M HCl
    - Add 3 g activated charcoal, mix for 5 minute and filter to remove charcoal
    - Add 3 g activated charcoal, mix for 5 minute and filter again
    - Store at room temperature in a dark bottle
2. Periodic acid solution

- 10 ml 7% acetic acid
- 20 µl 50% periodic acid

3. Decolourised Schiff's reagent

- 100 mg sodium metabisulphite
- 6 ml Schiff's reagent
- Incubate at 37°C until colourless
- Prepared fresh for every assay

Bradford Assay

- Bradford reagent is diluted 1:5 with dH2O

4-20% Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)

1.Sample application buffer

- 2% sodium dodecyl sulphate (SDS)
- 10% glycerol
- 0.01% bromophenol blue

The reagents were dissolved in dH2O and stored at room temperature.

2. 30% Bis/Acrylamide

- 30 g acrylamide

- 0.8 g bis

The reagents were dissolved in 100 ml dH2O and stored in a dark bottle at 4°C

3. 1.5 M Tris-buffer with 0.1% SDS, pH 8.8

- 1.5 M Tris
- 0.1% SDS

The reagents were dissolved in dH2O, adjusted to pH 8.8 and stored at 4°C

4. Spacer gel buffer

- 0.25 M Tris
- 0.2% SDS

The reagents were dissolved in dH2O and adjusted to pH 8.8. This buffer was stored at 4°C

5. 10% AMPS

- 10% AMPS dissolved in dH2O

Stored at 4°C

6. Tank buffer

- 0.025 M Tris
- 0.19 M glycine

- 0.1% SDS

The reagents were dissolved in 5 litres of dH2O, adjusted to pH 8.8 and stored at room temperature

4-20% SDS-PAGE

1. 4% light solution

   - 0.8 ml 30% Bis/Acrylamide
   - 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8
   - 3.7 ml dH2O
   - 30 µl 10% AMPS
   - 5 µl TEMED

2. 20% heavy solution
   - 4 ml 30% Bis/Acrylamide,
   - 1.5 ml 1.5 M Tris-buffer with 0.1% SDS, pH 8.8,
   - 0.5 ml dH2O,
   - 30 µl 10% AMPS
   - . 5 µl TEMED

Using a serological pipette, 2.3 ml of the 4% light solution is pipetted and thereafter 2.3 ml of the 20% heavy solution is pipetted into the same pipette. A single air bubble is passed through the solution to create the 4-20% gradient.

Periodic acid Schiff (PAS) gel staining

1. Schiff's reagent

- 1 g pararosanniline hydrochloride dissolved in 200 ml boiling distilled water, with constant stirring
- Cool solution to 50°C on bench and 20 ml 1 M HCl
- Cool to 25°C and add 1 g sodium metabisulphite and leave in the dark for 12-24 hours
- Add 2 g activated charcoal, mix for 1 minute and filter
- . Store at 4°C in the dark

2 50% ethanol

- 50 ml ethanol
- 50 ml distilled water

3. 1% periodic acid and 3% acetic acid

- 50% periodic acid
- 3% acetic acid

Slot blot

1. 20× SSC

- 175.3 g sodium chloride
- 88.2 g tri-sodium citrate

The reagents are added to 1 litre dH2O, adjusted to pH 7 and stored at 4°C

2. 4× SSC

- 200 ml 20× SSC, pH 7
- 800 ml dH2O

This buffer was stored at 4°C

Western blotting and slot blot

3. 1× TBST

- 1.21 g Tris-HCl
- 8.76 g sodium chloride
- 0.5 g Tween 20

The reagents are added to 1 litre dH2O, adjusted pH 8 and stored at 4°C

Salt azide buffer (0.2 M NaCl and 0.02% NaN3)

- 11.69 g NaCl
- 0.2 g NaN3

5% and 10% DMEM

- 5% DMEM
- 50 ml FBS
- l NEAA
- l of 200x PenStrep
- 0.5ml of 100x L-glutamine

The reagents are made up to 50 ml with a DMEM and L-glutamine solution

10% DMEM

- 5.0 ml FBS
- 0.50 ml NEAA
- 0.25 ml of 200x PenStrep
- 0.5ml of 100x L-glutamine
- The reagents are made up to 50 ml with a DMEM and L-glutamine solution

Appendix 2: Consent form, information sheet and ethics

Study Title; The effect of reduction, trypsin digestion and de-glycosylation of salivary mucins in the inhibition of the human immunodeficiency virus type 1

Patient information sheet

Dear Patient

We are doing a study on the role of mucins (the "sticky" portion of mucus in your saliva) in the inhibition of HIV-1 virus infection. This research study will be conducted by Miss Tsetse under the supervision of Professor Anwar S. Mall in the Department of Surgery, Old Main Building, Groote Schuur Hospital.

HIV transmission via the oral route remains rare despite the detection of the AIDS virus in the oral cavity. One apparent reason for this rare transmission is the presence of antiviral factors such as the innate inhibitory molecules, mucins, and soluble proteins which are found in saliva. It has been reported that mucins can inhibit the activity of HI virus.

We aim to determine the structural variants of mucins which are essential for the inhibition of HIV-1 infection and the effect reduction and trypsin digestion of this "sticky" mucus has in inhibiting the HIV-1 virus.

We require 40 mls of Saliva. You will be expected to spit into an Eppendorf tube after chewing a piece of plastic parafilm (You are required not to swallow it). This is a once off thing. You will be asked to rinse your mouth prior to the procedure, to prevent food contamination of the saliva. Prior to saliva collection, an HIV test will be conducted. We are planning on recruiting 50 HIV negative patients who are attending the maternity wards of Groote Schuur Hospital, Cape Town, South Africa as part of HAART service that is normally offered to pregnant women in maternity wards across the country. In doing so, we ensure our cohort is HIV negative. We hope that the findings of this study will give us a better understanding of the role saliva (in particular mucins) plays in the inhibition of HIV-1.

Please note the following:

1. Informed consent will be taken before sample collection takes place.

2. We will need to take your folder number (not your name) from which we will get the details of your age, gender and ethnicity. We require participant's folder numbers such that in the case of an anomaly in our results, or in case the results we get from certain participants do not fit the trend observed, we can be able to fully justify/attribute the differences in findings. We do hope having access to the participant's folder will give us an understanding if there is a link between any medical conditions the participants might have prior to providing their samples and our results.

3. All this information will remain strictly confidential and we promise that your saliva samples will not be used for any other purpose, except for the purpose of this study.

4. You have every right to not participate in this study. If you do not wish to take part in this study, it will not affect the treatment you receive.

5. If you agree to participate in this study and change your mind at a later date, it will be fine. You will be removed from this study and your decision will not affect the treatment and care you receive.

6. It is possible that we will publish the findings of this study. In this case, your name will not be mentioned and will still remain confidential.

7. We must emphasize that all samples collected for this study will not carry your name for reference.

8. The samples will be stored in the laboratory for re-analysis.

If there are any questions, please ask your doctor or call the number below. If you think of any questions at a later date, please contact us on the number below.

Professor A. Mall

Tel; 021 406 6168/6227

HREC TEL; 021 406 6338Room; E52.26, Old Main Building, GSH

Request for saliva samples for biochemical analysis

Research laboratory

Division of General Surgery

OMB Groote Schuur Hospital

UCT, Medical School, Observatory, 7925

Tel; 021 406 6168/6227Fax; 021 448 6461

Please fill in all the information requested

Folder No; _____

HIV status; _____

Weeks at testing; _____

For laboratory use only

Date received; _____

Computer Index No; _____

Please note that your HIV status is recorded on this form.

1. I give permission that my saliva can be taken for research purposes in the investigation of HIV AIDS.

2. I give permission that a portion of saliva can be stored indefinitely for:

a) Possible re-analysis

b) Research purposes subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.

3. I authorize / do not authorize my doctor(s) (delete where not applicable) to provide relevant clinical details to the researchers.

4. All of the above information has been fully explained to me in a language I understand, and all my questions were answered.

Participant's signature;_____ Date; _____

Turnitin Originality Report
tstell001:CHAPTER_ONE_turnit_in_.docx by Ellis Tsetse
From For TurnItIn Submission (99e58091-4ed3-452e-ae8f-f82d0b847550)

- Processed on 15-Mar-2019 15:01 SAST
- ID: 1093826759
- Word Count: 29002

Similarity Index
22%
Similarity by Source
Internet Sources:
6%
Publications:
9%
Student Papers:
18%

Signature Removed

Anwar Suleman Mall